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Quality Control and Quality Assurance Procedures for Level 1 Health Effects Bioassays

Litton Bionetics, Inc., Kensington, MD

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QUALITY CONTROL AND QUALITY ASSURANCE PROCEDURES FOR LEVEL 1 HEALTH EFFECTS BIOASSAYS

by

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Quality Assurance	Health Effects	
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This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

ABSTRACT

The purpose of this project was to develop a program of quality control (QC) and quality assurance (QA) for the EPA Level 1 environmental assessment health effects bioassays to ensure that all data collected are of known and documented quality sufficient to meet their intended use. This document is designed as a companion document for the "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests" (EPA-600/8-81-023). The Level 1 procedures manual presents procedures for the collection, pretest handling, and bioassay of environmental samples as well as a brief discussion of QC and QA procedures associated with the Level 1 environmental assessment biological testing program. This report expands upon the QC and QA material presented in the Level 1 procedures manual and provides a comprehensive approach to QC and QA for the Level 1 health effects bioassays. The objective of this project was to provide a model framework of QC and QA procedures, that when followed, would ensure that all data collected, stored, reported or used by the U.S. Environmental Protection Agency would be scientifically valid, defensible, and of known precision and accuracy.

This QC/QA manual presents information on the use of laboratory performance audit samples, documentation requirements for sample identification and handling, and provides quality control procedures and documentation requirements for the Level 1 health effects bioassays. These bioassays include the Ames _Salmonella_/microsome mutagenesis assay, the Chinese hamster ovary cell toxicity assay, the rabbit alveolar macrophage toxicity assay and the _in vivo_ rodent toxicity assay.

The procedures and sample documentation forms presented in this document are designed to serve only as models for QC and QA procedures and should not be viewed as the only acceptable approach to QC and QA.

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CHAPTER 1

INTRODUCTION

1.1 LEVEL 1 QUALITY CONTROL AND QUALITY ASSURANCE PROGRAM OVERVIEW

The purpose of this document is to serve as a supplement to the EPA/IERL-RTP Level 1 environmental assessment biological testing manual, second edition (1). This manual is a guide to quality assurance (QA) and quality control (QC) considerations necessary for the performance of the Level 1 health effects bioassays. The objectives of this report are to:

1. Supplement the Level 1 procedures manual with respect to the description of standard operating procedures for quality control steps associated with the health effects bioassays.

2. Describe the role and use of laboratory quality assurance audit samples prepared to establish uniformity in laboratories conducting Level 1 health effects bioassays.

3. Describe the test material sampling and processing documentation required for the Level 1 environmental assessment program.

4. Provide a description of the protocol steps which require documentation under the good laboratory practices (GLP) regulations of the U.S. Food and Drug Administration (FDA).

5. Provide a series of sample forms which can be used in fulfilling the documentation requirements.

Recently, the U.S. Environmental Protection Agency (EPA) has published requirements for good laboratory practices (2,3), patterned after the specifics of the good laboratory practices (GLP) regulations of the U.S. Food and Drug Admininstration (FDA) (4). GLPs require that all phases of a biological assay be documented and reviewed by the assigned individuals. These regulations also specify that documentation of the adequacy of test materials, facilities, and personnel be made. Once the adequacy of the facility and personnel have been documented, the GLPs require the following basic information:

1. A detailed study design describing the steps involved in the bioassay. The study design (protocol) must be supported by laboratory Standard Operating Procedures (SOPs).

2. A complete set of raw data with support documentation of medium and reagent batch or lot number, source, and expiration date. All data must be entered in a permanent record and signed and dated by the investigator. Methods for analysis must also be defined and justified.

3. A report which provides all data collected in the study. The contents of the report must define the study conditions and must be consistent with both the original study design and the raw data.

This manual outlines most of the quality control and documentation steps required to meet EPA and FDA GLPs. QC and QA procedures are not intended to restrict test performance or improvements in study designs but are critical in order to maintain uniformity of data generation, documentation and interpretation. Each Level 1 health effects assay is divided into steps. For each step, the major operations are identified and the quality control steps needed are listed. Those steps which need to be documented are identified and sample forms are provided to assist in the documentation procedure. Alternate forms can be accomodated to suit specific laboratory procedures or protocol modifications. GLP's require that all items entered onto forms be signed or initialed and dated. Any alterations to the permanent record must be initialed, dated and the reason for alteration noted.

Coded test materials are available for determining laboratory proficiency. The nature of these samples and rationale for their use are described in a separate document (5). Directions for the use of proficiency samples are summarized in this document.

1.2 REFERENCES

1. Brusick, D.J., and R.R. Young. IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests. EPA-600/8-81-024, NTIS PB 82-228966, Litton Bionetics, Inc. Kensington, MD, October 1981, 150 pp.

2. Proposed Health Effects Test Standard for Toxic Substances Control Act Test Rules; Good Laboratory Practice Standards for Health Effects. Federal Register, Part II, volume 44, No. 91, May 1979 and Part IV, volume 44, No. 145, July 1979.

3. Guidelines for Registering Pesticides in the United States: Proposed Good Laboratory Practice Guidelines for Toxicology Testing. Federal Register, volume 45, No. 77, April 1980.

4. DHEW Food and Drug Administration. Nonclinical Laboratory Studies; Good Laboratory Practice Regulations. Federal Register, volume 43, No. 247, pp. 59986-60020, Part II, December 22, 1978.

5. Litton Bionetics, Inc. Evaluation of Test Materials for Health and Ecological Effects Bioassay Proficiency Testing. EPA Contract No. 68-02-2681, Technical Directive 113, Kensington, MD, February 1982, 97 pp.

CHAPTER 2

LABORATORY PERFORMANCE AUDIT SAMPLES

2.1 QUALITY ASSURANCE AUDIT SAMPLES

Laboratories conducting Level 1 health effects bioassays or intending to initiate health effects studies are encouraged to document their proficiency in the health effect bioassays by testing laboratory performance audit samples. These samples will be supplied to the laboratory as coded samples. The laboratory will perform Level 1 health effects tests using procedures described in the Level 1 health effects procedures manual (Second Edition).

The results of the tests will be reported to the EPA/IERL reference laboratory. EPA and the reference laboratory will review the data and prepare a report evaluating the performance of the qualifying laboratory based upon the following:

1. Demonstration of an understanding of and compliance to procedures described in the Level 1 health effects manual (Second Edition).

2. The laboratory work and data meet Quality Control and Quality Assurance requirements described in this document.

3. The final data, data interpretation, and audit sample ranking match the test data base for each bioassay developed by the EPA/IERL reference laboratory on the same audit sample.

Coded laboratory assessment samples have been prepared to submit to laboratories wishing to ensure Level 1 testing proficiency. The audit substances have the following characteristics:

1. Physical properties similar to those of natural samples, but with defined compositions to ensure reproducible preparation.

2. Samples are stable when stored under normal ambient conditions.

3. The audit samples exhibit a full range of bioassay responses from no detectable toxicity (or mutagenicity) to high toxicity (or mutagenicity).

4. A set of audit samples maybe prepared with different levels of difficulty assigned to each set, permitting different levels of discrimination in the quality assurance or certification process.

At the present time, coded audit samples have been developed with the characteristics described. More information on the audit sample program and the availability of laboratory audit samples may be obtained from:

Technical Support Staff
Industrial Environmental Research Laboratory
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

The results of the tests conducted by the requesting laboratory will be evaluated against the results established for each sample by a reference laboratory with an extensive data base for test results with the audit samples in the appropriate Level 1 bioassays. The reports submitted by the requesting laboratory will also be evaluated for compliance with Level 1 protocols and reporting requirements. Tables 1 and 2 provide examples of items reviewed in the auditing process for two of the Level 1 health effects bioassays. When the audit is completed, a full report will be prepared by the auditing laboratory and submitted to the laboratory which conducted the Level 1 testing.

The samples will be shipped to the requesting laboratory as coded samples for laboratory testing. A set of safety data sheets identifying the compounds and outlining safe handling procedures will be sent in sealed envelopes to the laboratory's safety officer to be opened only in the event of accidental spill or human exposure. These envelopes are to be returned unopened under normal circumstances. An example of the information provided in these sheets is shown in Table 3.

In addition to a narrative analysis of the laboratory's performance, each bioassay data set will be compared with that of the reference laboratory using specially prepared forms. Sample forms for the Ames assay and the CHO toxicity assay are shown in Tables 4 and 5.

TABLE 1. AMES MUTAGENESIS ASSAY QUALITY ASSURANCE AUDIT CRITERIA

Study Design	Quality Control	Report
Cell Maintenance	Cell Maintenance	Results
Preparation of Strains	Preparation of Strains	Protocol
Culture Media	Culture Media	Tables
Metabolic Activation	Metabolic Activation	Graphs
Preparation of Test Sample	Preparation of Test Sample	Summary
Assay Conditions	Data Acceptance Criteria	Organization
Data Evaluation Criteria	Data Evaluation Criteria	

TABLE 2. CHO CLONAL TOXICITY ASSAY QUALITY ASSURANCE AUDIT CRITERIA

Study Design	Quality Control	Report
Cell Maintenance	Cell Maintenance	Results
Prep. of Test Cultures	Prep. of Test Cultures	Protocol
Prep. of Test Sample	Prep. of Test Sample	Tables
Treatment of Cells	Treatment of Cells	Graphs
Data Evaluation	Data Acceptance Criteria	Summary
	Data Evaluation Criteria	Organization

TABLE 3. SAMPLE MATERIAL SAFETY DATA SHEET

Code Number: ________________

Name of Compound: 2-Aminoanthracene

Synonyms: 2-Anthramine
2-Anthracenamide

Molecular Weight: 193.26

Molecular Formula: N-C14-H11

Toxic Effects Class: CARCINOGEN

Chemical Class: Aromatic amine

Toxicity Data: orl-rat TDLo: 45mg/kg/30DI
skn-ham TDLo: 6000mg/kg/55WI
LD50 orl (24hr)-mice: 1.5 gram

NSC #: 400535

Chemical Abstract Service (CAS) Registery Number: 613138

NIOSH Toxic Substances List #: CA92750

Special Warnings:
- Handle as a carcinogen. Avoid all contact with this chemical. Any overt human exposure should be immediately treated by washing exposed skin with soap and water. Exposure to the eye should be treated by washing with copious amounts of water.
- Dispose of any wastes as a carcinogen.
- Report all human exposures or spills to supervisory personnel and health-care personnel.
- Medical care should be available to any exposed personnel.

TABLE 4. AMES ASSAY INTER-LABORATORY QUALITY AUDIT DATA COMPARISON

CHEMICAL CODE NUMBER ____

	QUALIFYING LAB	REFERENCE LAB
MAXIMUM DOSE		
DOSE RANGE		
SOLVENT		
MUTAGENICITY		
STRAIN RESPONSE		
MEC (MUTAGENICITY)		
REV./PLATE AT MEC		
FOLD INCREASE AT MEC		
DOSE RESPONSE		
MUTAGENICITY CATEGORY		

TABLE 5. CHO ASSAY INTER-LABORATORY QUALITY AUDIT DATA COMPARISON

CHEMICAL CODE NUMBER ____

	QUALIFYING LAB	REFERENCE LAB
MAXIMUM DOSE		
DOSE RANGE		
NUMBER DOSES TESTED		
SOLVENT		
CLONING EFFICIENCY (VEHICLE CONTROL)		
EC_{50}		
TOXICITY CATEGORY		

CHAPTER 3

DOCUMENTATION REQUIRED FOR SAMPLE IDENTIFICATION AND HANDLING

3.1 RATIONALE

In order to properly evaluate samples and reach meaningful conclusions regarding their biological properties, information on the collection, processing, and shipping of environmental samples must be transmitted with the samples.

The forms provided in this document are proposed for use by the on-site sampling manager and the EPA Project Officer. The first form, "Level 1 Sample Collection Form" (Table 6), details the manner in which the samples are collected and processed before shipment to the biological testing laboratory. This information is used in determining the emission rate of tested samples and aids in estimating potential environmental exposure. The second form, "Level 1 Sample Processing Form" (Table 7), permits selection of the type of bioassays and sample preparation procedures to be performed on the sample. This form also identifies the minimum quantity of test material requested for each type of bioassay.

The next three forms provide a continuity of documentation for test materials at the laboratory performing the biological testing. The "Chemical Repository Worksheet" (Table 8) provides a synopsis of information on a test sample. The "Chemical Repository Log" (Table 9) is used to document withdrawal of test material from the repository and to coordinate test material usage. The "Test Material Disposal Log", (Table 10) documents the disposal of test materials that are no longer under study.

3.2 SAMPLE FORMS (TABLES 6-10)

The following set of forms are adequate to provide sufficient information about an environmental sample to perform Level 1 health effects bioassays.

TABLE 6. LEVEL 1 BIOASSAY SAMPLE COLLECTION FORM

I. SAMPLE INFORMATION

1. Sample No. ______________________ Collection Date ______________
2. Sampling Site ____
3. Field Sampling Manager (on-site) _______________________________
4. Contractor ______________________ Contract No. _________________
5. Project Officer ________________ Program Name __________________
6. Source Sampled ____
7. Discharge Rate of Source (Volume/Time) __________________________
8. Quantity Sampled/Units ____
9. Sample Description (liquid, slurry, solid, extract, appearance, etc)

   ____

   ____
10. Other Information as Applicable

    Collection temp. _______________ Sampling location _____________

    pH ____________________________ Sampling technique _____________

    Other ____

II. HANDLING & SHIPPING

1. Describe Sample Treatment Prior to Shipping (e.g., transfers, extractants, stored neat, grinding, solvents used) ________________

   ____
2. Field Storage and Shipping Conditions

Container	Temperature	Light
☐ Amber Glass	☐ Ambient	☐ Shield from light
☐ Polyethylene Bottle	☐ Refrigerate (0 to 4°C)	
☐ Coated Bag or Bottle	☐ Freeze (-20°C)	
☐ Teflon or Tedlar Bags	☐ Dry Ice	
☐ Other ______________		

3. Time in Storage/Time in Shipping ________________________________
4. Sample Shipped to ____

   ____
5. Mode of Shipping/Carrier _______________________________________
6. Comments ____

   ____

TABLE 7. LEVEL 1 BIOASSAY SAMPLE PROCESSING FORM

I. SAMPLE IDENTIFICATION

A. Sample No. ____________ Collection Date ____________

B. Bioassay Contractor ____________ Program Manager ____________

C. Project Officer ______________________

D. Brief Sample Name ______________________

II. SAMPLE TYPE AND LAB PROCESSING

Basic Type	Subtype	Processing
Solid	☐ Solid Granular ☐ Slurry, > 5% Solids ☐ Particulates from Filter ☐ Filter and Particulates ☐ Other ________	☐ Grind to <5 μm or ☐ <3/8 in. ☐ Extract Particulates with Organic Solvent ☐ Remove Particulate from Filter ☐ Prepare Water Leachate ☐ Test as is ☐ Other ________
Liquid	☐ Suspensions, < 5% Solids ☐ Aqueous ☐ Nonaqueous ☐ Extract ☐ Condensate ☐ Other ________	☐ Concentrate ________ ☐ Solvent Exchange ☐ Evaporate to Dryness ☐ Filter ☐ Test as is ☐ Other ________
Gas	☐ Pressure Collection ☐ Vacuum Collection	

III. BIOASSAYS REQUESTED AND QUANTITY REQUIRED

Health Effects	Solid	Aqueous Liquid	Nonaqueous Liquid
☐ Mutagenesis (Ames)	2.0 g	5 ml	5 ml
☐ Macrophage toxicity (RAM)	0.1 g	45 ml	2 ml
☐ Rodent Cell Clonal toxicity (CHO)	0.1 g	45 ml	2 ml
☐ Mice *in vivo* toxicity (WAT)	10.0 g	50 ml	20 ml
Ecological Effects			
☐ Freshwater fish	10 kg	40 L	1 L
☐ Freshwater invertebrate	0.5 kg	2 L	0.2 L
☐ Freshwater algae	0.25 kg	1 L	0.1 L
☐ Marine fish	10 kg	40 L	1 L
☐ Marine invertebrate	2 kg	8 L	0.8 L
☐ Marine algae	0.25 kg	1 L	0.1 L
Terrestrial Ecological Effects			
☐ Plant stress ethylene (PSE) (1,365 liters gas)	---	---	---
☐ Plant Root Elongation (RE)	2.5 kg	10 L	---
☐ Insect Toxicity	0.5 g	20 ml	10 ml
☐ Other tests (explain on back)			

IV. RECEIPT OF SAMPLE

Date of Receipt ____________ Received by ____________

TABLE 8. CHEMICAL REPOSITORY WORKSHEET

A. GENERAL INFORMATION

1. SPONSOR/PROJECT NO. ________________

2. SAMPLE IDENTITY ________________

3. ASSAY NO. ________ SPONSOR ID NO. ________

4. STORAGE CONDITIONS ________________

5. SPECIAL WARNINGS/PRECAUTIONS ________________

6. DATE RECEIVED ________________

7. SAMPLE TO BE RETURNED TO SPONSOR? YES ______ NO ______

8. PRETEST SAMPLE PREPARATION ________________

B. COORDINATION OF SAMPLE USE

9. LIMITED AMOUNT OF SAMPLE? YES ___ NO ___

10. IF SAMPLE AMOUNT IS LIMITED, IDENTIFY TESTS AND ANTICIPATED QUANTITY OF TEST MATERIAL REQUIRED

TEST	AMOUNT GM OR ML
________	________
________	________
________	________

C. CHEMICAL/PHYSICAL DATA (PROVIDED BY RECEIVING TECHNICIAN)

11. PHYSICAL DESCRIPTION ________________

12. GROSS WEIGHT (COMPOUND + CONTAINER) ________________

13. INTEGRITY OF CONTAINER ________________

D. SAMPLE DISPOSITION

14. ASSAYS COMPLETED (DATE OF MEMO) ________________

15. RETENTION SAMPLE TAKEN

AMOUNT ________ DATE ________ TECH. INITIALS ________

16. DISPOSITION OF UNUSED SAMPLE:

GROSS WEIGHT (COMPOUND + CONTAINER) ________________

DATE ________ INITIALS ________

METHOD OF DISPOSAL: I. DRUM ________

II. RETURN TO CLIENT ________

III. INCINERATE ________

TABLE 9. CHEMICAL REPOSITORY LOG

ASSAY NO. ________ SAMPLE IDENTITY: _______ INITIAL AMOUNT: ________

	REMOVED	DATE	TECH.	PURPOSE	LAB
1.					
2.					
3.					
4.					
5.					
6.					
7.					

SAMPLE USED UP: DATE _____________ TECH. ____________________________

TABLE 10. TEST MATERIAL DISPOSAL LOG

ASSAY NO.	COMPOUND	DISPOSAL METHODS	RETENTION SAMPLE	TECHNICIAN INITIALS	DATE

CHAPTER 4

QUALITY CONTROL PROCEDURES AND DOCUMENTATION REQUIREMENTS FOR THE AMES SALMONELLA/MICROSOME MUTAGENESIS ASSAY

4.1 INTRODUCTION

The purpose of this chapter is to serve as a supplement to the EPA Level 1 Manual (1) and is a guide to quality control and quality assurance considerations as they apply to the performance of the Ames *Salmonella*/microsome mutagenesis assay outlined in the following flow diagram (Figure 1). In the following sections, each of the assay steps is individually developed by identifying the separate operations involved, describing the quality control functions associated with the operations, and by outlining the necessary documentation. In most cases, the documentation requirements will be satisfied by completing data reporting forms similar to those provided as samples at the end of this assay description.

The objective of the Ames *Salmonella*/microsome mutagenesis assay is to determine whether a test material is mutagenic. For samples that are mutagenic, the minimum concentration of test material that produces a positive (mutagenic) response is determined. Once identified, the minimum effective concentration (MEC) is used to rank the mutagenic activity of the test material using evaluation criteria presented in this chapter.

4.2 CULTURE OF SALMONELLA STRAINS (STEP 1A)

4.2.1 Operations

1. Cells from the stock maintenance plate are removed and added to nutrient broth (Oxoid II Broth) in an Erlenmeyer flask.

2. The flask is shaken at 150 rpm at 37°C until the cell density reaches early stationary phase (1×10^9 cells/ml). This should take approximately 16 hours.

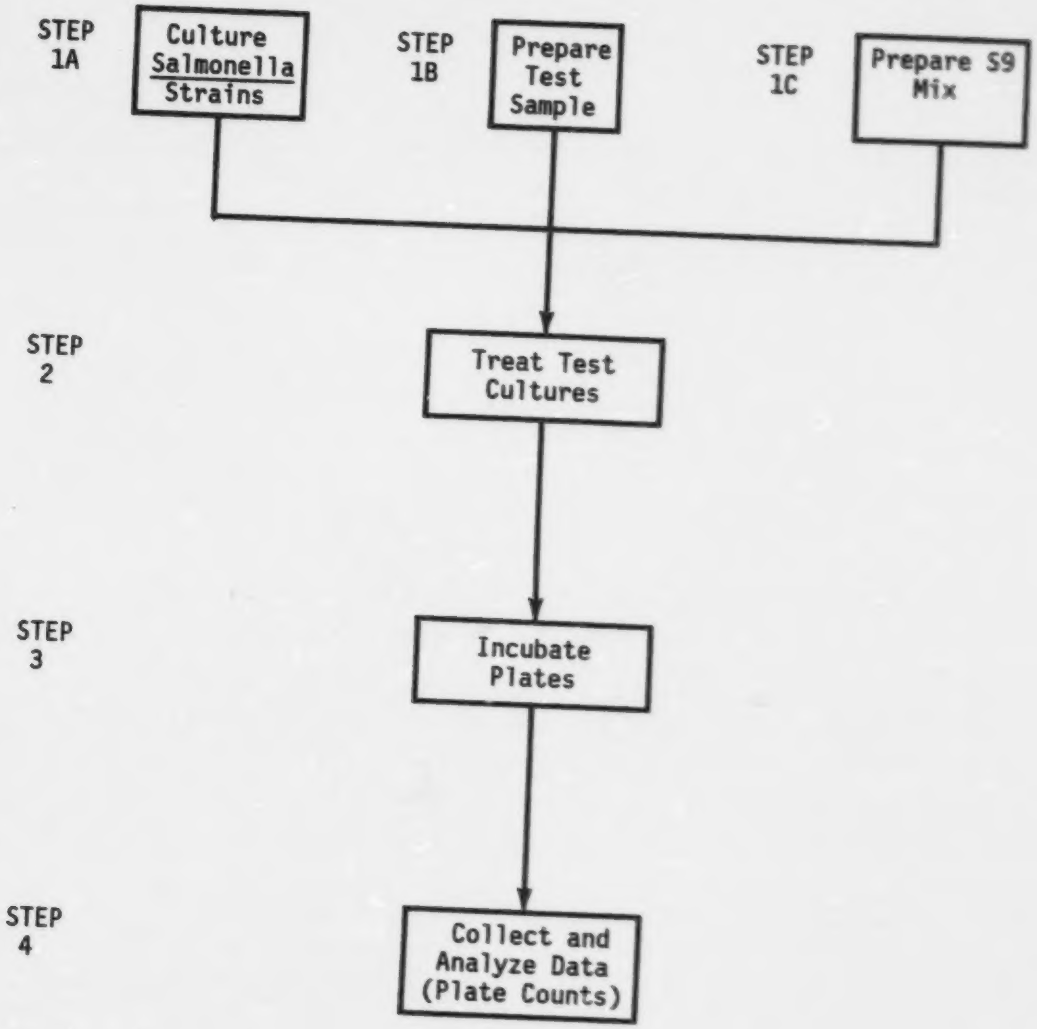
3. Cells are held at +4°C and used within four hours.

4.2.2 Quality Control

1. Nutrient broth must be checked for sterility before inoculation.

2. Each strain must be checked each day for ampicillin resistance, crystal violet sensitivity, spontaneous reversion frequency, and contamination.

3. Viable cell density must not be lower than about 1×10^9 cells/ml in the test culture.


STEP 1A
Culture Salmonella Strains
STEP 1B
Prepare Test Sample
STEP 1C
Prepare S9 Mix
STEP 2
Treat Test Cultures
STEP 3
Incubate Plates
STEP 4
Collect and Analyze Data (Plate Counts)

The *Salmonella* plate assay is divided into four steps. The procedures and quality control functions needed to adequately perform these steps are described in the following sections.

Figure 1. Ames assay steps.

4.2.3 Documentation (Ames forms 1 through 4)

1. The responsible technician fills out the Salmonella Strains Quality Control Data Form (Ames form 1) as the quality control characteristics of each tester strain are determined daily. Each QC entry must be initialed and dated and constitutes a part of the permanent laboratory record.

2. Each culture should be numbered and identified in the test.

3. Culture media, reagent and incubator logs are maintained that identify the quantity supplier, lot number and expiration data of each component used (Ames form 2).

4. Records are maintained of each autoclave run using Ames form 3. If time/ temperature recorders are used, retain the charts in a permanent file.

5. Daily recordings of temperature are maintained with each incubator (Ames form 4).

4.3 PREPARATION OF TEST SAMPLE (STEP 1B)

4.3.1 Operations

1. Solid samples are ground to homogeneous, fine powders or, if supplied as a fine powder, are used without further preparation. Soluble solids are dissolved so as to give the desired dose in a volume of 200 µl or less. Insoluble solids are suspended and diluted in the desired solvent. Solubility tests are recorded. Solvents, in order of preference, are: water, dimethyl sulfoxide, ethanol, N,N-dimethyl formamide, acetone and toluene.

2. Liquids are added directly by volume. Samples are processed and tested as recommended in the Level 1 manual. Nonaqueous samples and resin extracts are solvent exchanged to dimethylsulfoxide (DMSO) if possible.

3. The scope of testing may be reduced if the amount of sample is limiting. The number of strains used, the number of doses or the number of replicate per dose may be reduced in number. A minimum test matrix should include strains TA-98 and TA-100.

4.3.2 Quality Control

1. Upon receipt, each sample is given a unique identification number, is described physically, and is stored as specified (or as appears appropriate).

2. All chemical and physical transformations of the sample are described in a manner that allows the final preparation to be related in quantity and/or concentration to the originally supplied sample.

3. A description of the test sample distribution in and reaction with the test media must be provided.

4. A sterility check of the test material is made before Ames testing is initiated.

4.3.3 Documentation (Ames form 5)

1. The responsible technician fills out the appropriate sections of Ames form 5 for the sample type at the time of the assay. Each form is dated and initialed, and constitutes a part of the permanent laboratory record.

2. Records from the responsible unit doing test sample preparation and manipulations are obtained and maintained with the bioassay data record.

3. The culture media and reagent logs are maintained that identify the quantity, supplies, lot number, and expiration data of each component used. See Section 4.2.

4. Records are obtained and maintained documenting removal of aliquots of test material and positive controls from the chemical repository and the use of the analytical balance. Calculations are recorded for preparing dosing stocks of test material and positive controls.

4.4 PREPARATION OF S9 MIX (STEP 1C)

4.4.1 Operations

1. Male, Sprague-Dawley rats are ordered so as to be (8 to 10 weeks old at the time of use). Animals are quarantined two weeks prior to use. Animals should be between 180 and 250 gm at the time of use.

2. Animals are induced with Aroclor 1254 in corn oil (200 mg/ ml) (2). The Aroclor is administered in one intraperitoneal injection at a dosage of 0.5 mg/kg of body weight. After five days the animals are sacrificed using a guillotine and are then immediately exsanguinated. Food, but not water, is withheld during the last 12 hours.

3. The livers are aseptically removed and homogenized as described in the Level 1 manual. After homogenization in 0.15 M KCl the suspension is centrifuged for 10 minutes at 9,000 x g at 4°C.

4. The supernatant (S9 fraction) is decanted into small vials frozen in dry ice and stored at -75° to -80°C.

5. The arylhydrocarbon hydroxylase (AAH) enzymes are measured spectrophotofluorometrically and serve as an indication of the relative P450/P448 enzyme activity (3).

6. The amount of protein (mg/ml) is measured using the Lowry Method (4).

7. The metabolic activation capability of the S9 is measured by using it in an Ames test over a range of S9 concentrations to activate 2-aminoanthracene (2.5 μg/plate) and benzo(α)pyrene (5 μg/plate). The concentration of S9 which gives maximum reversion is used for routine testing (5). Other reference chemicals that require metabolic activation may be used in place of those mentioned above.

8. Prepare, sterilize and store solutions as described in the following section.

9. Prepare complete S9 reaction mix on day of use by combining components as described in the following section.

4.4.2 Quality Control

1. Rats are examined for visable signs of sickness during quarantine, induction, and prior to sacrifice. Animals are identified by ear tag numbers.

2. Sterile techniques are used in excising the livers and in preparing the S9 fraction. Tissue, glassware and solutions are kept cold.

3. Sterility of S9 preparation is determined. Reaction mixture may be filter sterilized if needed.

4. Arylhydrocarbon hydroxylase enzyme activity is determined for each S9 lot using a spectrophotofluorometer.

5. The protein content (mg protein/ml homogenate) is determined for each S9 lot with the Lowery technique using a spectrophotometer.

6. The ability of each S9 lot to metabolically activate chemicals to mutagenic forms is determined. Optimum S9 concentrations for each lot is determined.

7. QC tests of each S9 lot are reviewed by the laboratory supervisor.

8. The pH meter is calibrated with reference pH solutions before use.

9. Use of S9 reaction mix should not exceed 6 hours at 0°C.

4.4.3 Documentation (Ames forms 6 through 14)

1. Details of S9 homogenate production are recorded using Ames forms 6 and 7. These forms will become part of the permanent record for the lot of S9.

2. Preparation of activation system components is recorded using Ames forms 8, 9, 10 and 11.

3. Each time complete S9 reaction mix is prepared, the work is recorded on Ames form 12.

4. Quality control tests of each S9 lot for AAH enzymes, protein content, activation capability for reference mutagens and sterility are recorded on Ames form 6 and summarized using Ames forms 13 and 14.

5. Media and reagent logs are maintained that identify the quantity, supplier, lot number and expiration date of each component used. See Section 4.2.

6. Equipment and incubator logs are maintained.

4.5 FORMULATION OF ACTIVATION SYSTEM

4.5.1 S-9 Reaction Mix Components

	Components	M.W.	Concentration/ml S9 Mix
1.	Nicotinamide adenine dinucleotide phosphate (NADP)	801	4 µmoles
2.	D-glucose-6-phosphate (G6P)	283	5 µmoles
3.	Magnesium chloride ($MgCl_2$)	203.3	8 µmoles
4.	Potassium chloride (KCl)	74.6	33 µmoles
5.	Sodium phosphate buffer (PB) pH 7.4		100 µmoles
6.	Organ homogenate (S9 fraction)		100 µliters

Make-up following stock solutions:

0.2 M NADP	Millipore filter
0.2 M G6P	Millipore filter
0.4 M $MgCl_2$	Sterilize by autoclaving
1.65 M KCl	Sterilize by autoclaving
0.2 M Na Phosphate buffer pH 7.4	Sterilize by autoclaving

Solution A: 7.5 ml aliquots of 0.2 M G6P are dispensed into sterile vials and kept frozen at -80°C until used.

Solution B: 6 ml aliquots of 0.2 M NADP are dispensed into sterile vials and kept frozen at -80°C until used.

Solution C: Mix the following and store at 4°C.

0.4 M $MgCl_2$	20 ml
1.65 M KCl	20 ml
0.2 M PB pH 7.4	500 ml
Sterile Distilled H_2O)	315 ml

4.5.2 Formulation of S-9 Reaction Mix

Solution D: Core Reaction Mixture

Make the core reaction mixture (Solution D) by adding:

0.2 M G6P (Solution A)	2.5 ml	
0.2 M NADP (Solution B)	2.0 ml	
Solution C.	85.5 ml	
	90.0 ml	Filter Sterilize

Solution D should be prepared each day and all components should be kept ice cold before and after mixing.

4.5.3 S9 Reaction Mixture (Solution E)

To 90.0 ml of Solution D add 10 ml of Organ homogenate (S9) to make up 100 milliliters of S9 reaction mixture. If needed, filter sterilize through a 0.45 µm membrane filter. Both Solution D and Solution E should be kept cold (on ice) before and after mixing. S9 reaction mixture should be used the same day it is prepared. DISCARD UNUSED PORTION.

4.6 TREATMENT OF TEST CULTURES (STEP 2)

4.6.1 Operations

1. Plate incorporation version of the Ames assay is performed by adding test components as indicated in the schematic diagram (Figure 2).

2. Test materials and controls are assayed in duplicate (two plates per dose) both with and without S9 metabolic activation mix.

3. *Salmonella typhimurium* strains to be used include TA-1535, TA-1537, TA-98 and TA-100.

4. Overlay agar, phosphate buffer, histidine and biotin stocks are prepared.

5. Bottom agar (selective medium) is prepared and dispensed into 15 mm x 100 mm sterile petri dishes.

6. Test cultures, test material and S9 reaction mix are prepared as described in Steps 1A, 1B and 1C.

7. Overlay tubes containing test material (or solvent or positive control), test organism, S9 reaction mix (or phosphate buffer) and overlay agar are prepared. Overlay agar is held at 45° ± 2°C before use.

8. Contents of overlay tubes are poured onto plates containing bottom agar.

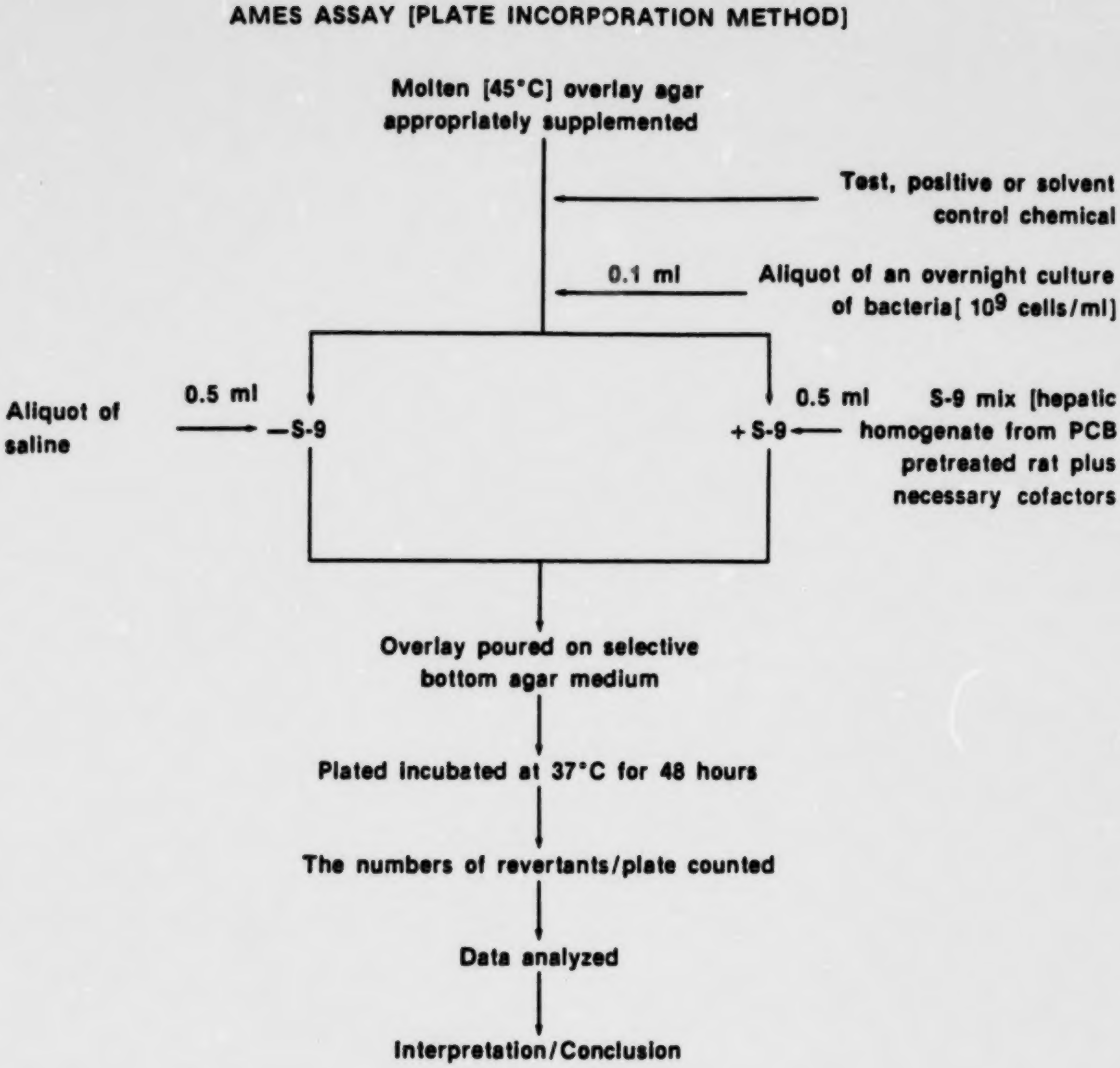

AMES ASSAY [PLATE INCORPORATION METHOD]
Molten [45°C] overlay agar appropriately supplemented
Test, positive or solvent control chemical
0.1 ml
Aliquot of an overnight culture of bacteria[10⁹ cells/ml]
Aliquot of saline
0.5 ml
—S-9
+S-9
0.5 ml
S-9 mix [hepatic homogenate from PCB pretreated rat plus necessary cofactors
Overlay poured on selective bottom agar medium
Plated incubated at 37°C for 48 hours
The numbers of revertants/plate counted
Data analyzed
Interpretation/Conclusion

Figure 2. Ames *Salmonella* microsome mutagenesis assay.

4.6.2 Quality Control

1. Sterile techniques are used handling stock cell cultures, test material and other ingredients.

2. At least two cultures are prepared for each test concentration unless test material is limiting.

3. Preliminary toxicity or limited range-finding mutation assays may be performed at the discretion of the study director.

4. Maximum applied concentrations for the Ames assay are 5000 µg/plate for solids and 200 µl/plate for liquids.

5. Sterility of each lot of culture medium and reagents are determined before use. Bottom agar plates are preincubated at 37°C for one to two days prior to use to check for sterility.

6. Steps in the assay should be performed in as short a period of time as possible.

4.6.3 Documentation (Ames forms 9 and 15 through 18)

1. The appropriate forms for the preparation of phosphate buffer (Ames form 9), histidine/biotin stock solution (Ames form 15), overlay agar (Ames form 16) and bottom agar (Ames form 17) are filled out.

2. General media and reagent logs are maintained. See Section 4.2.

3. Autoclave, incubator and other equipment logs are maintained. See Section 4.2.

4. The Plate Test Identification Sheet (Ames form 18) is completed when a test is performed.

4.7 INCUBATION OF PLATES (STEP 3)

4.7.1 Operations

1. Plates are placed in an incubator at 37°C once the overlay medium has solidified.

2. The incubation period is 48 hours, unless there is evidence of growth inhibition. If inhibition is evident, the incubation period may be extended to 72 hours.

4.7.2 Quality Control

1. If the incubator is not vented, it is recommended that control and treated plates be placed in separate incubators.

4.7.3 Documentation

1. A record of incubator temperature is maintained. See Section 4.2.

4.8 COLLECTION OF MUTAGENESIS DATA (STEP 4)

4.8.1 Operation

1. Plates are removed from the incubator and colonies counted by hand or with an automatic colony counter.

2. Data is collected and tabulated.

4.8.2 Quality Control

1. The automatic colony counter is calibrated daily using a test pattern dish according to manufacturer's recommendations and to standard sensitivity settings developed for use in each laboratory.

2. Plates removed from the incubator may be held up to eight hours at room temperature or up to 48 hours at +4°C before counting.

3. Final data tables are proofed against raw data collected on Ames form 19.

4.8.3 Documentation (Ames form 19)

1. All data entered into standard forms must be dated and initialed by technicians. The form constitutes a part of the permanent laboratory record for the assay.

2. All data must be reviewed, initialed and approved by supervisor.

3. Equipment logs are maintained.

4. If data entry into a computer is used, the technician and the file name of the data set are documented.

4.9 ASSAY EVALUATION AND RANKING CRITERIA

4.9.1 Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test chemical, the surviving population on the treatment plates is essentially the same as that on the negative control plate. At high concentrations, the surviving population is presumed to be reduced. An adequate protocol normally employs several doses ranging over two or three log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

4.9.2 Dose-Response Phenomena

The demonstration of dose-related increases in mutant counts is possibly the most important criterion in establishing a mutagenic effect. A factor that might modify dose-response results for a mutagen would be the selection of doses. If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test chemical may kill any mutants that are induced, and the compound will not appear to be mutagenic.

4.9.3 Control Tests

Positive and negative control assays are conducted with each experiment and consist of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays. Negative controls consist of the test compound solvent in the overlay agar together with the other essential components for both nonactivation and activation portions of the assay. The negative control plates for each strain give a reference point to which the test data are compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens. The recommended spontaneous revertant range limits for Salmonella strains are given in Table 11.

TABLE 11. ACCEPTABLE SPONTANEOUS REVERTANTS PER PLATE

STRAIN	REVERTANTS/PLATE
TA-1535	20 ± 10
TA-1537	15 ± 10
TA-98	50 ± 25
TA-100	150 ± 75

4.9.4 Evaluation Criteria for Ames Assay

Because the procedures used to evaluate the mutagenicity of the test chemical are semiquantitative, the criteria used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets are evaluated using the following criteria:

Strains TA-1535 and TA-1537--

If the solvent control value is within the normal range, a chemical that produces a positive dose response over three concentrations with the maximum increase equal to three times the solvent control value for strains TA-1535 and TA-1537 is considered to be mutagenic.

Strains TA-98 and TA-100--

If the solvent control value is within the normal range, a chemical that produces a positive dose response over three concentrations with the maximum increase equal to twice the solvent control value for strains TA-98 and TA-100 is considered to be mutagenic. For these strains, the dose-response increase should start at approximately the solvent control value.

Reproducibility--

If a chemical produces a response in a single test that cannot be reproduced in one or more additional runs, the initial positive test data lose significance. If the initial test is negative, it should be repeated after one week for confirmation with strains TA-98 and TA-100. If the results are positive, repeat studies should be performed over a narrower concentration range with strains showing positive results in the initial test to identify the minimum effective concentration (MEC).

The proceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria are applied to the majority of situations and are presented to aid those individuals not familiar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

4.9.5 Criteria for Ranking Samples in the Ames Assay

The goal of EPA Level 1 Ames testing is to rank source streams by relative degree of genetic toxicity (mutagenicity). Samples are first identified as mutagenic or nonmutagenic by the criteria in Section 4.9.4 above and then ranked using the mutagenicity categories presented in Table 12 below. The lowest concentration giving a positive response in any strain, with or without metabolic activation, is identified as the minimum effective concentration (MEC) for that sample. The mutagenicity of the sample is evaluated as high (H), moderate (M), low (L) or nondetectable (ND) according to the evaluation criteria developed in the Level 1 manual (1) and summarized below. Samples with no detectable activity at the maximum applicable dose (MAD) are ranked nondetectable (ND).

TABLE 12. AMES ASSAY MUTAGENICITY EVALUATION CRITERIA (1)

Mutagenic Activity	Solids (MEC in μg/plate)	Liquids[a] (MEC in μl/plate)
High (H)	<50	<2
Moderate (M)	50-500	2-20
Low (L)	500-5000	20-200
Not Detectable (ND)	>5000	>200

[a] Concentration of organic extracts is based upon organic content (μg organics per plate and not volume (μl extract per plate) of sample tested.

4.10 REFERENCES

1. Brusick, D.J., and R.R. Young. IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests. EPA-600/8-81-024, NTIS PB 82-228966, Litton Bionetics, Inc., Kensington, MD, October 1981, pp. 150.

2. Freireich, E.J., *et al*. Quantitative comparison to toxicity of anticancer agents in mouse, rat, dog, monkey, and man. Cancer Chemotherapy Reports, 50(4):219-244, 1966.

3. Nebert, D.W. and H.V. Gelboin. Substrate inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. J. Biol. Chem., 243:6242-6249, 1968.

4. Lowry, O.H., *et al*. Protein measurement with folin phenol reagent. J. Biol. Chem., 193:265-275, 1952.

5. Ames, B.N., J. McCann, and E. Yamasaki. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Res., 31:347-364, 1975.

4.11 SAMPLE FORMS FOR THE AMES *SALMONELLA*/MICROSOME MUTATION ASSAY

The following set of data recording and standard procedure documentation forms are adequate to provide the necessary information to audit the EPA Level 1 Ames *Salmonella*/microsome assay.

AMES FORM 1. SALMONELLA STRAINS QUALITY CONTROL DATA FORM

DATE ______________

		QC Points						
Strain	Culture #	A	B	C	D	E	Remarks	Initials

QC Point	Strain Characteristic
A	Crystal Violet (+ = sensitive, - = nonsensitive)
B	Ampicillin (+ = sensitive, r = resistant)
C	Adequate Spontaneous Frequency (+ = yes, - = no)
D	Adequate Viable Cell Titer (+ = yes, - = no)
E	Contaminated (+ = yes, - = no)

AMES FORM 2. MEDIA AND REAGENT LOG

Product ____________________ Date ____________________

Assigned Lot No. ______________ Tech ____________________

Expiration Date ______________

Component	Amount	Supplier	Lot No.	Exp. Date

AMES FORM 3. AUTOCLAVE RUN LOG

Autoclave Number ________________ Date ____________________

Clean Plug Screen ______ (Initials)
Check Recorder Pen _____ (Initials)

Run Number	Name of Operator	Sterilizing		Type of Load		Contents
		Time (Min)	Pressure (lbs)	Liquid	Dry	

AMES FORM 4. TEMPERATURE RECORD FOR INCUBATORS

TEMPERATURE RECORD FOR THE INCUBATOR NO.__________
I.D. NO.__________

DATE	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEPT	OCT	NOV	DEC
1												
2												
3												
4												
5												
6												
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AMES FORM 5. PREPARATION FORM FOR CHEMICAL STOCKS

Compound Identification
Tech/Date Prepared
Solvent/Lot No.
Description

Stock Concentrations Prepared According to SOP No.

Working Notes and Observations While Preparing Stock Solutions

The Test was Conducted According to [indicate by ✓]
[ ] SOP No.[s]:
[ ] Procedure Alteration

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AMES FORM 6. S-9 PRODUCTION WORKSHEET

S-9 DATA	
LOT NUMBER	DATE ANIMALS RECEIVED/SUPPLIER/P.O.#
SPECIES/SEX/STRAIN	DATE ANIMALS INDUCED/SYSTEM [LOT#]
VOLUME S-9/DATE DISPENSED	DATE SACRIFICED
TISSUE WEIGHT	DATE HOMOGENIZED/BUFFER [LOT#]
STERILITY	HOMOGENIZED BY

LOWRY PROTEIN DETERMINATION		
STANDARD [BOVINE SERUM ALBUMIN]		SAMPLE
µg/ml	O.D.	O.D.
120		µg/ml
240		DILUTION
360		mg/ml [S9]
480		TESTED BY
600		DATE

BIOLOGICAL ACTIVITY: MUTAGENESIS TITRATION DATA							
		REVERTANTS/PLATE AT VARIOUS CONCENTRATIONS OF S-9					
STRAIN	MUTAGEN	-C	.01ml	.02ml	.05ml	.1ml	.15ml
TA-98	2.5µg 2-AA						
TA-98	µg						
TA-100	2.5µg 2-AA						
TA-100	µg						
DILUTED BY	DATE		COUNTED BY			DATE	

BENZO(a) PYRENE [CYTOCHROME P448/450] ASSAY							
LOT#	F.U.	A.	C.F.	C.F. xA	mg/ml PROTEIN	S.A.	R.I.
TESTED BY			DATE				

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AMES FORM 7. PREPARATION OF KCl (FOR HOMOGENIZATION)

Tech ______________________________ Date ______________

WEIGHT	GM	SUPPLIER/LOT #
paper/pan	________	
KCl	________	______________
total weight	________	

VOLUME	ML
distilled H_2O	________

assigned lot # ____________________

Autoclave for 15 minutes at 121°C.

AMES FORM 8. PREPARATION OF GLUCOSE-6-PHOSPHATE (SOLUTION A) FOR S9 MIX

Tech ______________________________ Date ______________

WEIGHT	GM	SUPPLIER/LOT #
paper/pan	________	
Glu-6-PO_4	________	______________
total weight	________	

VOLUME	ML
distilled H_2O	________

assigned lot # ____________________

Filter using 0.45µm filter unit and store at -80°C.

Label SOLUTION A.

AMES FORM 9. PREPARATION OF PHOSPHATE BUFFER

Tech ______________________________ Date ____________________

WEIGHT	gm	gm	SUPPLIER/LOT #
paper/pan	________	________	
sodium phosphate dibasic (Na_2HPO_4)	________		________
sodium phosphate monobasic (NaH_2PO_4)		________	________
total weight	________	________	

VOLUME	ML
distilled water	________
pH	________
checked by	________
assigned lot #	________

pH should be 7.4.

Autoclave for 15 minutes at 121°C and store in refrigerator after cooling.

AMES FORM 10. PREPARATION OF NADP STOCK (SOLUTION B) FOR S9 MIX

Tech ______________________ Date ______________________

WEIGHT	MG	SUPPLIER/LOT #
paper/pan	______________	______________
NADP	______________	______________

VOLUME	ML
distilled water	______________

Assigned Lot # ____________

Filter using .45μm filter unit. Store at -80°C.

Label SOLUTION B.

AMES FORM 11. PREPARATION OF SOLUTION C FOR S9 MIX

Tech ______________________ Date ______________________

VOLUME	ml	SUPPLIER/LOT #
$MgCl_2$ (0.4 M)	______________	______________
KCl (1.65 M)	______________	______________
Phosphate buffer (pH 7.4)	______________	______________
Sterile Dist. H_2O	______________	______________

Final Volume ____________

Assigned Lot # ____________

Label SOLUTION C.

All compounds are sterile. Store at +4°C.

AMES FORM 12. COMBINATION OF COMPONENTS FOR S9 MIX

Tech ______________________________ Date ______________________________

SOLUTION	VOLUME	ml	LOT #
A	GLU-6-PO_4		
B	NADP		
C	SOLUTION C		
	S9 HOMOGENATE		
	FINAL VOLUME		

Filter if necessary and use. Store on ice. Discard unused portion.

AMES FORM 13. SPECIFICATIONS FOR RAT LIVER S9

Lot Number: ______________________________

Induced: ______________________________

Animal Species: ______________________________

Strain: ______________________________

Date Prepared: ______________________________

Protein: ______________________________ mg/ml

Benzo(α)pyrene Hydroxylase Activity:

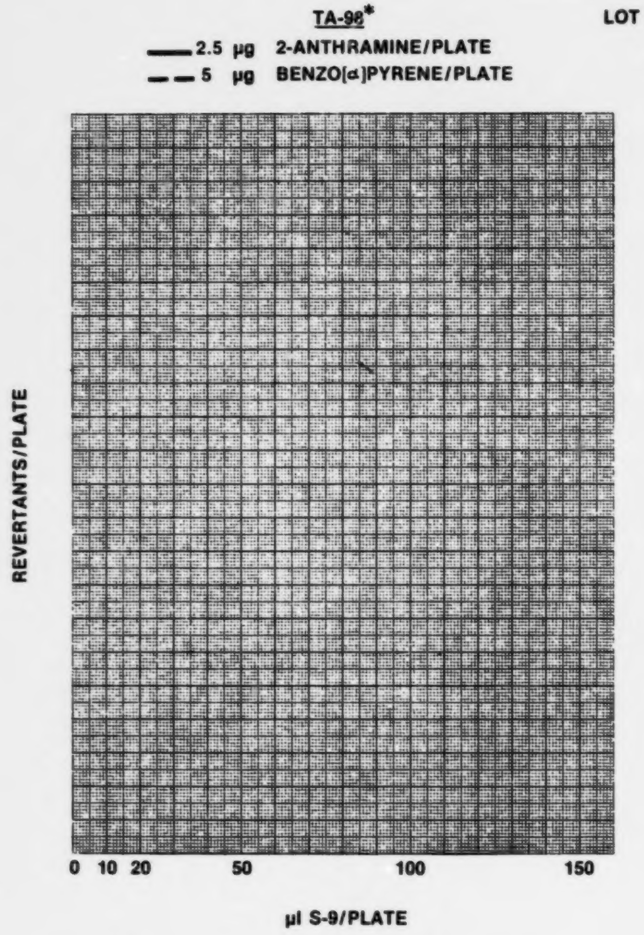
______ nmol hydroxybenzpyrene/20 minutes/mg protein.

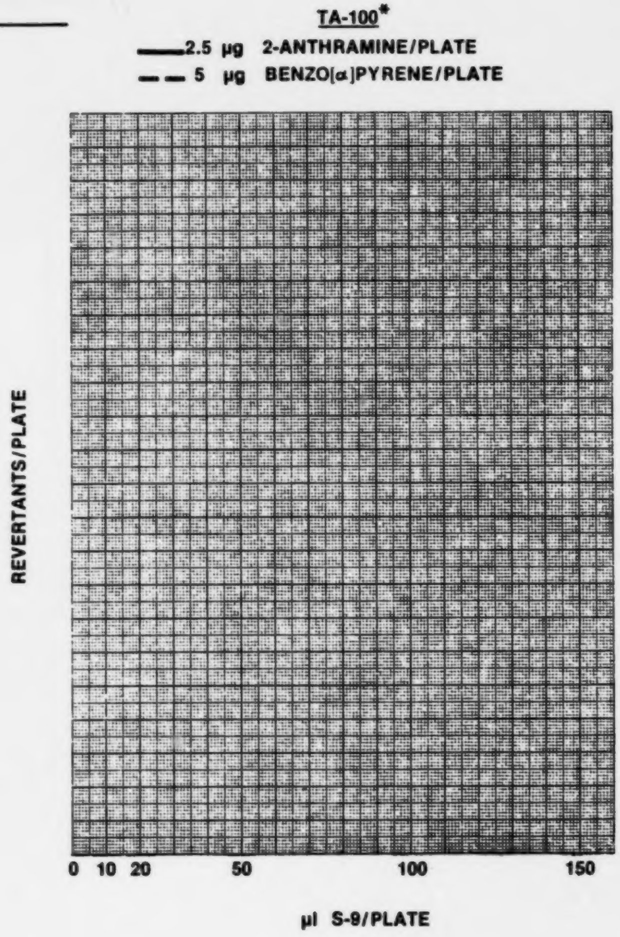
$$\underline{\qquad\quad}\,\% = \frac{\text{IRL-}\underline{\qquad\qquad}\text{ hydroxybenzpyrene}}{\text{noninduced S9 hydroxybenzpyrene}} \times 100$$

Sterility: ______________________________

AMES FORM 14. MUTAGENESIS TITRATION

LOT #________


TA-98*


TA-100*

*450 μl CORE REACTION MIXTURE/PLATE

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AMES FORM 15. PREPARATION OF HISTIDINE/BIOTIN STOCK

Tech ______________________________ Date ___________________________

Stock Solutions

Histidine and Biotin

Histidine	7.8 mg
Biotin	12.2 mg
H_2O	100 ml

Filter sterilize using 0.45μ filter

WEIGHT	gm	gm	SUPPLIER/LOT #
paper/pan	__________	__________	
histidine	__________		______________
biotin		__________	______________
total weight	__________	__________	

VOLUME	ML
distilled water	__________
assigned lot #	__________

Heat biotin at 80°C until dissolved, then add histidine. Filter using .45μm filter unit. Store in the refrigerator at +4°C.

AMES FORM 16: PREPARATION OF OVERLAY AGAR

Tech ____________________ Date ____________________

WEIGHT	gm	gm	SUPPLIER/LOT #
paper/pan	________	________	
purified agar	________		________
sodium chloride [NaCl]		________	________
total weight	________	________	

VOLUME	ML	LOT #
distilled water	________	
Hist/Biot	________	________
assigned lot #	________	

Hist/Biot added prior to dispensing agar and after agar is sterilized.

OVERLAY AGAR

REGULAR (FOR SALMONELLA)

H_2O	1 liter
NaCl	5 grams
Agar	6 grams
Histidine-biotin stock	100 ml

NOTE: Autoclave water, NaCl and agar at 121°C for 15 minutes and add sterile histidine/biotin stock.

AMES FORM 17. PREPARATION OF BOTTOM AGAR

Tech ______________________ Date ____________________

STOCK SOLUTIONS (Each solution is autoclaved at 121°C for 15 minutes).

SOLUTION AND LOT #	INGREDIENT	AMOUNT	TARE (gm)	WEIGHT (gm)	TOTAL (gm)	SUPPLIER	LOT #
I. Agar							
Lot # ____	Agar	15 gm	___	___	___	______	__
	Water	1000 ml					
II. 40% Glucose							
Lot # ____	Glucose	40 gm	___	___	___	______	__
	Water	100 ml					
III. 50x Vogel-Bonner Salts							
Lot # ____	Warm Water	670 ml					
	$MgSO_4 \cdot 7H_2O$	10 gm	___	___	___	______	__
	Citric acid monohydrate	100 gm	___	___	___	______	__
	K_2HPO_4	500 gm	___	___	___	______	__
	$NaHNH_4PO_4 \cdot 4H_2O$	175 gm	___	___	___	______	__

Solution III: Add salts in order indicated and allow each salt to dissolve completely before adding the next. Bring to 1 liter when done.

PREPARATION OF BOTTOM AGAR

Combine Solution I (agar) with 50 ml of solution II (40% glucose) and 20 ml solution III (50x VB Salts) then dispense into plates.

Solution I ____ ml ____ lot #
Solution II ____ ml ____ lot #
Solution III ____ ml ____ lot #

Assigned Lot # ______
Plates __________

AMES FORM 18. PLATE TEST IDENTIFICATION SHEET

BUFFER LOT #: ____________________

DOSE UNITS: A-µg/plate, B-µl/plate, C-time units, D-sq. inch, E-other ______

PERFORMED BY: 1. Plate set up by ____________________

2. Chemical added by ____________________

3. Indicator organism added by ____________________

4. S-9 added by ____________________

5. Overlay poured by ____________________

WATER BATH TEMPERATURE ____________________

OVERLAY TUBES: HIST/BIOT, SPECIAL ____________________

DATE/TIME PERFORMED ____________________ TECH __________

DATE/TIME PLATES REMOVED FROM INCUBATOR ______________ TECH __________

DATE/TIME PLATES COUNTED ____________________ TECH __________

COMMENTS ____________________

- -

RETURN THIS SHEET TO YOUR SUPERVISOR FOR FILING AFTER PLATES ARE REMOVED FROM THE INCUBATOR.

LOT NUMBERS:

SOLVENT(S)

DMSO ____________________ Agar plates ____________________

Acetone ____________________ Overlay agar ____________________

Other ____________________

AMES FORM 19. AMES PLATE ASSAY DATA SHEET

①Chemical Name or Code [1-80] ______

②Solvent [1-20] ______

Test Dates M / D / Y [21-26] M / D / Y [27-32]

Date Completed M / D / Y [33-38]

Concentration Units ______ [39-40]

☐ 41

1 = -S9 Mix Only
2 = +S9 Mix Only

S9 Lot# [42-51] ______

Table# ☐ 52

Client ______ Date Recvd. ______ Recvd. By ______

Physical Description ______ Assay# ______

Activation System Employed ______ Safety# ______ Tech. ______

Other Considerations ______

		Indicator Organisms																	
		TA-1535			TA-1537			TA-1538			TA-98			TA-100					
	Concentration	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	Solvent																		
	Solvent																		
	Positive																		
	Positive																		
-S9 Mix																			

	Solvent																		
	Solvent																		
	Positive																		
	Positive																		
+S9 Mix																			

Evaluation/Conclusion ______ Submitted By ______

LBI-0184R 6/81 a

CHAPTER 5

QUALITY CONTROL PROCEDURES AND DOCUMENTATION REQUIREMENTS FOR THE CHINESE HAMSTER OVARY (CHO) CELL CLONAL TOXICITY ASSAY

5.1 INTRODUCTION

The EPA Level 1 Manual defines the scope of the Environmental Assessment Program and provides detailed assay procedures and data analysis for all phases of its implementation. The following sections provide supplementary material specifically related to the quality control and documentation of the CHO Clonal Toxicity Assay.

For convenience in presentation of the various operations, the CHO Clonal Toxicity Assay is organized into six steps. A flow diagram of the steps is provided in Figure 3. In each of the following sections, one step is developed by identifying the separate operations involved, giving the quality control functions associated with the operations, and by defining the necessary documentation. In most cases, the documentation requirements will be satisfied by completely filling out data recording forms similar to those provided at the end of this chapter.

The objective of the CHO assay is to determine the concentration of a test material that reduces the colony-forming ability of cultured Chinese hamster cells by 50 percent after a 24-hour exposure. This concentration is calculated or estimated graphically from the assay data and is referred to as the EC_{50} value.

5.2 CELL CULTURE MAINTENANCE (STEP 1)

5.2.1 Operations

1. CHO-K1 cells are available from the American Type Culture Collection, Rockville, MD.

2. Monolayer cultures of CHO-K1 cells are incubated at 37°C in a humidified ambient air atmosphere containing five percent CO_2. Stock cultures are maintained in 250 cm^3 plastic tissue culture flasks. During incubation, caps of the flasks are loosened to permit gas exchange.

3. Laboratory stock cultures are routinely subcultured to lower density cultures to maintain a continuous line of metabolically active and dividing cells. Subculture is accomplished by rinsing a cell monolayer with Dulbecco's phosphate buffered saline (Ca^{++} and Mg^{++} free), treatment with either a 0.25 percent trypsin solution or 0.1 percent trypsin plus 0.01 percent EDTA solution, trituration to form a cell suspension, and seeding aliquots of cell suspension into new culture flasks with fresh medium.

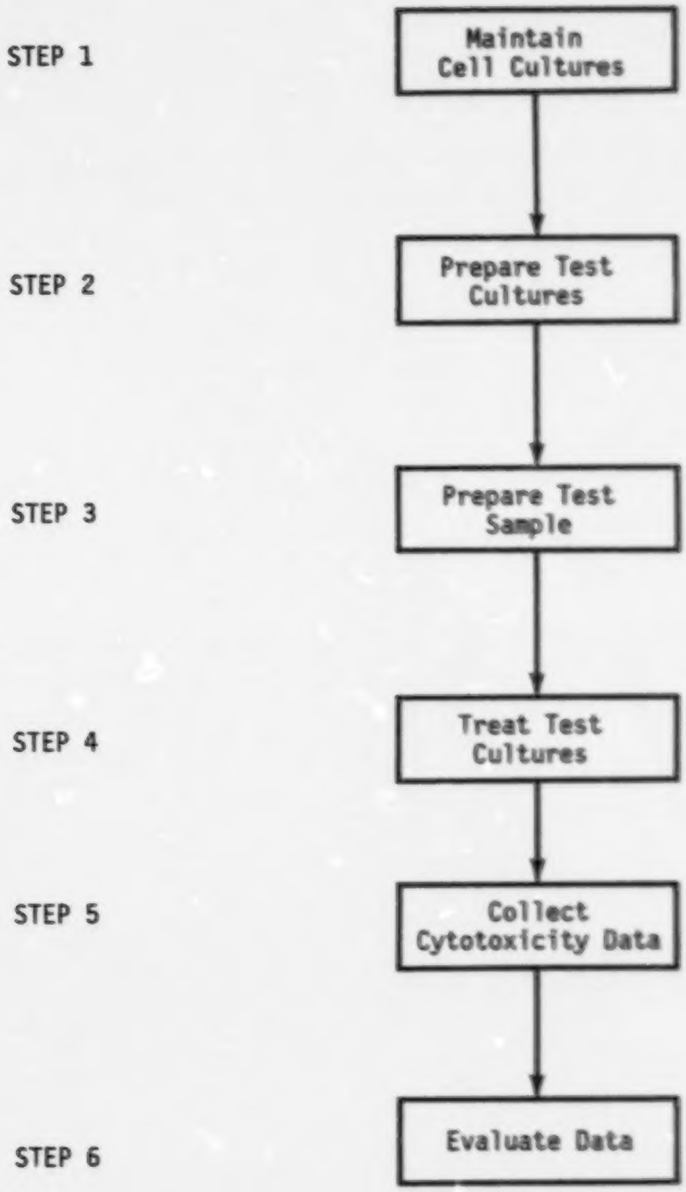

STEP 1
Maintain Cell Cultures
STEP 2
Prepare Test Cultures
STEP 3
Prepare Test Sample
STEP 4
Treat Test Cultures
STEP 5
Collect Cytotoxicity Data
STEP 6
Evaluate Data

Figure 3. CHO clonal toxicity assay steps.

4. Reserve cell stocks are maintained by freezing cell suspensions and holding in liquid nitrogen.

5. Culture medium is prepared as needed and stored under refrigeration (+4°C). Ham's nutrient mixture F12 is routinely used and supplemented with ten percent fetal bovine serum, 2 mM L-glutamine, and antibiotic agents. Protection against contamination is provided by routinely supplementing the F12 with penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (Fungizone) (0.5 μg/ml). Kanamycin, at 100 μg/ml, may also be added for extra protection against contamination.

5.2.2 Quality Control

1. The laboratory stock cultures are routinely subcultured every two to four days, before (or when) complete confluency is achieved. Overgrown cultures contain dead cells and cells in abnormal metabolic states that can lead to divergent cell types and unrepeatable responses to test materials. Cultures should not exceed 3×10^5 cells per cm^2 of culture surface area.

2. Continuous laboratory stocks are replaced every three to four months with new cultures started from frozen stocks. This procedure helps to control against diverging cell cultures that may result from unknown laboratory pressures.

3. Incubator temperature should be maintained at 37 ± 2°C. Stock cultures exposed to 40°C or higher should be discarded. Loss of heating for up to one day is not considered harmful.

4. The medium pH is partially controlled by the CO_2 level, which should be maintained at 5 ± 1 percent in the incubator or in closed culture flasks. Temporary excursions are permissible if the culture pH remains normal, as indicated by an orange-red to orange-yellow color due to the phenol red component. Cultures with purple medium ($pH > 7.8$) must be returned to normal pH within a few hours or be discarded. Yellow medium ($pH < 6.8$) is permissible, but not desirable, for up to 12 hours.

5. Serum lots should be tested for growth potential before purchase.

6. Stock cultures, media, and reagents should be routinely tested for bacterial or fungal contamination by streaking samples on nutrient and yeast extract agar plates (or the equivalent). Contaminated cultures or reagents are immediately discarded. Mycoplasma contamination, which is usually not obvious, should be assayed for about every two months by an experienced organization. Frozen stock cultures should be demonstrated to be free of Mycoplasma.

5.2.3 Documentation (CHO forms 1 through 4)

1. The cell identity, source, and other appropriate information about frozen cell stocks are recorded in a cell repository log (CHO form 1).

2. Records of laboratory stock culture growth and subculture frequency are maintained (CHO form 2).

3. Culture media and reagent logs are maintained that identify the quantity, supplier, lot number and expiration date of each component used (CHO form 3).

4. Daily recordings of temperature, humidity and CO_2 levels are maintained with each incubator and are periodically collected into a permanent record (CHO form 4).

5. A record of results obtained from sterility testing assays is maintained.

6. The responsible technician initials and dates the information recorded on forms such as those provided here.

5.3 PREPARATION OF TEST CULTURE (STEP 2)

5.3.1 Operations

1. A subconfluent stock culture is trypsinized (3 to 5 minutes and triturated with culture medium to form a single cell suspension.

2. A cell count is performed, using the trypan-blue dye-exclusion method. The percent viable cells and the number of viable cells per ml are determined for the cell suspension. An aliquot of cell suspension is then serially diluted with culture medium to a final density of 50 cells/ml.

3. A number of 60-mm culture dishes are seeded with 4 ml of cell suspension per dish (200 cells/dish) and the cultures are incubated for 6 to 18 hours to permit cell attachment. Alternate cell dilution and inoculation schemes are permissable.

5.3.2 Quality Control

1. Actively growing cells from subconfluent cultures are used to obtain high cloning efficiency in the assay and to help standardize the sensitivity of the cells to treatment with a test material.

2. Two separate dilutions in trypan blue are prepared and counted. Approximately 200 cells are counted per dilution to determine the cell density, counting each cell or cell group as one cell. The number of live (clear), dead (blue), and total live and dead cells for each dilution are recorded and averaged. The viable cell count should be greater than 90 percent. Cultures with lower viabilities are discarded. The trypsinization procedure must yield a predominantly single cell suspension. A low frequency of doublets and triplets is normally observed. Suspensions containing larger clumps of cells must be agitated further to dissociate the cell clumps or be discarded.

3. Equal numbers of cells are placed in each test culture dish. This procedure requires accurate pipeting from a cell suspension that is swirled frequently to maintain a homogeneous distribution of cells. Cells may also be pipetted from a spinner flask to ensure a homogeneous cell suspension.

5.3.3 Documentation (CHO form 5)

1. The responsible technician fills out the Preparation of Test Culture Form (CHO form 5) at the time of the assay. Each form is dated and initialed, and constitutes a part of the permanent laboratory record of operations and data.

2. The culture media, reagent and incubator logs are maintained. See Section 5.2.

5.4 PREPARATION OF TEST SAMPLE (STEP 3)

5.4.1 Operations

1. Solid samples are ground to homogeneous, fine powders or, if supplied as a fine powder, are used without further preparation. The particulate sample is weighed (usually 24 mg), vortexed dry to break up clumps and suspended in complete F12 medium with 10 percent fetal calf serum (heat inactivated for 30 minutes at 56°C). Insoluble particulate samples are preincubated for 20 hours in culture medium on a rocker platform at 37°C so as to leach water soluble components from the test material. Serial dilutions are performed with culture medium supplemented as described in section 5.2. Solvents, in order of preference, are: culture medium and dimethyl sulfoxide. Optional solvents are ethanol and acetone.

2. A 10x formulation of Ham's F10 medium is available commercially and is used for dosing aqueous test samples in order to avoid dilution of medium components. Both F12 and 1x F10, when supplemented in an identical manner, support the growth and cloning of CHO-K1 cells equally well. Aqueous samples and suspensions are used (as supplied) as one of the diluents for the 10x concentrate of F10 medium. Normally, 18 ml of sample is used to prepare 30 ml of test medium at the maximum concentration of 600 µl/ml. Serial dilutions are performed with 1x F10 medium containing 10 percent fetal calf serum.

3. Samples supplied in an organic solvent, usually dimethylsulfoxide (DMSO), are added to F12 culture medium. The highest test concentration is achieved by a 1:50 dilution. Lower concentrations are prepared by serial dilutions of test sample prepared in its vehicle, followed by 1:100 dilutions of the test sample into F12 medium. Normally, 0.6 ml of the test sample is consumed per assay.

4. Additional operations, such as grinding to a specified upper size limit, extractions, concentrations, and solvent exchange into DMSO may be specified for test samples. The final form will conform to one of the above descriptions.

5.4.2 Quality Control

1. Upon receipt, each sample is given a unique identification number, is described physically, and is stored as specified (or as appears appropriate).

2. All chemical and physical transformations of the sample are described in a manner that allows the final preparation to be related in quantity and/or concentration to the originally supplied sample.

3. Fine, particulate (insoluble) materials are placed in F12 medium to leach for 20 hours.

4. A description of the test sample distribution in and reaction with the test media must be provided. Any pH changes outside the normal culture range are measured.

5. The pH meter is calibrated with reference pH solutions before use.

6. If sample causes a drop in pH below 6.8 or an increase above 7.6, the sample is retested with the pH adjusted to pH 7.2. The pH may be adjusted to pH 7.2 using ultrapure HCl or NaOH.

5.4.3 Documentation (CHO forms 6 and 7)

1. The responsible technician fills out the appropriate sections of CHO forms 6 and 7 for the sample type at the time of the assay. Each form is dated and initialed, and constitutes a part of the permanent laboratory record of operations and data.

2. Records from the responsible unit doing test sample manipulations are obtained and maintained with the bioassay data record.

3. The culture media and reagent logs are maintained that identify the quantity, supplies, lot number, and expiration date of each component used. See Section 5.2.

4. Records are obtained and maintained documenting removal of aliquots of test material from the chemical repository, the use of the analytical balance and the pH meter.

5.5 TREATMENT OF TEST CULTURES (STEP 4)

5.5.1 Operations

1. The test cultures are removed from the incubator and the medium is aspirated away. Aliquots of 4 ml of test media are placed on each culture and the cultures are returned to the incubator.

2. Negative control cultures in triplicate are dosed with culture medium. Vehicle controls are also dosed using culture medium containing the level of solvent used in the test material cultures.

3. The cells are exposed for 24 hours at 37 ± 2°C in a humidified atmosphere containing 5 ± 1 percent CO_2. The test media is then removed, the cultures washed with saline, and fresh F12 culture medium is added. The cultures are returned to the incubator for six more days.

5.5.2 Quality Control

1. The cultures are exposed to the test samples approximately 6 to 18 hours after seeding. This time allows cell attachment but avoids significant cell division and the subsequent formation of two-cell colonies prior to treatment.

2. Three cultures are exposed to each test or control concentration to allow for contamination losses and to determine average responses.

3. The exposure conditions are standardized at 4 ml of test medium at 37°C for 24 hours to help ensure uniform results between laboratories. The maximum applied concentrations are standardized at 1000 µg/ml for solid materials, 600 µl/ml for aqueous samples, and 20 µl/ml for organic solutions.

4. Deviations in the temperature and pH from the acceptable range are recorded because this factor may be responsible for the observed toxicity.

5. The pH meter is calibrated with reference pH solutions before use.

5.5.3 Documentation (CHO form 8)

1. The responsible technician fills out the appropriate form (CHO form 8) at the time of operations. The form is dated and initialed, and constitutes a part of the permanent laboratory record for the assay.

2. The culture media and reagent logs are maintained that identify the quantity, supplies, lot number, and expiration date of each component used. See Section 5.2.

3. The incubator log is maintained. See Section 5.2.

4. The pH meter log is maintained.

5.6 COLLECTION OF CYTOTOXICITY DATA (STEP 5)

5.6.1 Operations

1. The medium is drained from the cultures and the surviving colonies are fixed with 70 to 100 percent ethanol and stained with Giemsa.

2. Colonies are counted by eye.

3. Raw data is manually recorded and, if a computer is used for data storage and processing, entered into the computer.

5.6.2 Quality Control

1. The cultures are not physically disturbed over the incubation period to avoid colony splitting or tailing. The incubator shelf must be vibration-free. Incubation is normally terminated six days after treatment and should not continue past seven days to minimize colony splitting.

2. Changes in the pH from the normal culture range are recorded at the end of the treatment period because this factor may be responsible for observed toxicity.

3. The pH meter is calibrated with reference pH solutions before use.

4. The ethanol fixative is added gently to avoid washing colonies off the dish surface.

5. Small colonies of approximately 50 cells or less are excluded from the colony count. This procedure minimizes the contributions of colony splitting and severely damaged survivors to the colony count.

6. Final data tables are proofed against raw data collected on CHO form 9.

5.6.3 Documentation (CHO form 9)

1. The responsible technician fills out CHO form 9 at the time of operations. The form is dated and initialed, and constitutes a part of the permanent laboratory record for the assay.

2. The reagent and pH meter logs are maintained. See Section 5.2.

3. Data entry into the computer and the file name of the data set are documented.

4. If plates are missing from a dose-set, the reason for the loss is identified: contamination (C), technical error (T) or plate not set up to conserve sample (S).

5. The entire assay is reviewed by the laboratory supervisor, and the data reporting form is initialed and dated.

5.7 DATA EVALUATION (STEP 6) (SAMPLE FORMS 10 AND 11)

5.7.1 Acceptance Criteria

To consider the assay results acceptable for an evaluation of the test material toxicity, the assay must have been performed within the guidelines given by the quality control items in the previous sections. In addition, the final results must meet the following criteria:

A. The average cloning efficiency of the CHO-K1 cells in the negative controls must be between 70 percent and 115 percent.

B. The distribution of colonies in the treated cultures is generally uniform over the dish surface.

C. The data points for test concentrations critical to the location of the EC_{50} are the averages of at least two treated cultures.

D. A sufficient number of test concentrations are available to clearly locate the EC_{50} within a toxicity region defined under "Toxicity Evaluation Criteria."

5.7.2 Toxicity Evaluation Criteria

The EC_{50} value represents the concentration of test material that reduces the colony-forming ability of CHO cells to 50 percent of the negative control or the appropriate vehicle control value. Toxicity data can be analyzed using statistical methods such as those developed by Garrett and Stack (1). Four statistical programs are available for a programmable calculator with magnetic-card capability. Data generated by the programs include the mean and standard deviation of replicate values probability (P) values, the estimated dose level at which a response attains a given magnitude (normally the EC_{50}), and the 95 percent confidence limits for a given EC_{50} determination. An alternate method to estimate EC_{50} values is to graphically fit a curve by eye through relative survival data plotted as a function of the logarithm of the applied concentration. Each data point normally represents the average of three culture dishes. In order to indicate the variability of the data, the high and low colony counts for each concentration are used to calculate the relative survivals, and the range is shown by a bar at the position of the plotted average. If no bar is shown, the variability is within the size of the symbol.

The toxicity of the test material is evaluated as high, moderate, low, or nondetectable according to the range of EC_{50} values defined in Table 13. In the case of organic extracts, the evaluation is based upon the weight (μg) of the extract per ml of culture medium. The EC_{50} of sorbent extracts of SASS train gas samples may also be converted to the equivalent volume of gas sampled (in dry standard cubic liters, meters, or feet).

TABLE 13. CHO ASSAY EVALUATION CRITERIA

Toxicity*	Solids (EC_{50} in μg/ml)	Aqueous Liquids (EC_{50} in μl/ml)	Nonaqueous Liquids[a] (EC_{50} in μl/ml)
High	<10	<6	<0.2
Moderate	10 to 100	6 to 60	0.2-2
Low	100 to 1000	60 to 600	2-20
Nondetectable	>1000	>600	>20

[a] Criteria for nonaqueous liquids are tentative and under evaluation.

5.8 REFERENCES

1. Garrett, N.E. and H.F. Stack. Cellular Toxicology Data Analysis Using a Programmable Calculator. Comput. Biol. Med., 10(3):153-167, 1980.

5.9 SAMPLE FORMS FOR THE CHO CLONAL TOXICITY ASSAY

The following set of data recording and standard procedure documentation forms are adequate to provide the necessary information to audit the EPA Level 1 Chinese hamster ovary cell (CHO) clonal toxicity assay.

CHO FORM 1. FROZEN CELL REPOSITORY RECORD

Cell Identity ____________	No. of Vials ____________
Original Cell Source ____________	Cell No./Vial ____________
Lab Culture Source ____________	Medium ____________
Assigned Identity Code ____________	Cryogenic Preservative, Identity and Concn. ____________
Freeze Date ____________	Location: Freezer ____________
Technician ____________	Column ________, Box ________

REMOVAL LOG

Vials	Date	Tech	Vials	Date	Tech	Vials	Date	Tech

CHO FORM 2. LABORATORY CELL STOCK RECORD

Cell Line ____________	Culture Code ____________
Stock Identity Code ____________	Culture Medium ____________
Thaw Date ____________	Technician ____________

Culture ID #	Date	Tech	Medium and Lot #	Comments

CHO FORM 3. MEDIA AND REAGENT LOG

Product ____________________ Date ______________

Assigned Lot No. ______________ Tech ______________

Expiration Date ______________

Component	Amount	Supplier	Lot No.	Exp. Date

CHO FORM 4. INCUBATOR LOG

I.D. No. ________

Chamber ________

Date	Time	Gauge Readings CO_2/Air	CO_2 Level %	CO_2 Pressure Line/Tank	Humidity	Temp °C	Changes	Tech Initials

CHO FORM 5. PREPARATION OF TEST CULTURES

Assay No. __________ Date ___________ Tech. ______________

1. Cell Suspension Preparation

Cell line and ID # ___________ # cultures used __________ Last subculture __________

PBS wash: # washes ______ Vol. ______ Supplier/Lot No. _______

Trypsin/EDTA addition: Time ____ Vol./dish ____ Lot No. _____

Medium addition: Time ____ Vol./dish ____

______ Medium (Lot No. _________) Total trypsinate vol. ________

Comments: ____

2. Verification of Single Cell Suspension (Optional)

Total single cells _________ Total cells _________

% Single cells = (Total single/total) x 100

_______________________ = _______________

Comments: ____

3. Cell Viability and Density Determination

Trypan blue: Concentration _____ Supplier ______ Lot No. _______

Hemacytometer dilution: _____ ml trypsinate + _____ ml trypan blue; dilution factor ____

		Live	Dead	Total
Count in ten 1 mm^2 areas:	Count 1	______	______	______
	Count 2	______	______	______
	Average:	______	______	______

% Viability = (live count/total count) x 100

________________________ = ________

Cell density = live count x dil. factor x 10^3

________________________ = ________

Comments: ____

CHO FORM 5 (Continued)

Assay No. __________ Date __________ Tech __________

4. Cell Dilution Sequence

Label	Cell Supension ml	Medium ml	Total Vol. ml	Dilution Factor	Cell Density
A (Trypsinate)					
B					
C					
D					
E					
F					

5. Test Culture Preparation

Dish size _____ No. of dishes ______ Cells seeded/dish _____

Dish Inoculum ____ ml Total inoculum needed: ____ ml of____ cells/ml.

Time seeded ______ Incubator ID _______ Conditions _____________

Medium ___________________ Lot No. ________________

Comments: ____

CHO FORM 6. PREPARATION OF PRIMARY TEST SAMPLE STOCK

Assay No. ________________ Date ________________ Tech ______________

Sponsor ____

1. Solid/Particulate Sample

Sample Identity ______________________ Sponsor ID No. __________

Description ____

Warnings/Precautions ____

Date Received ________________ Storage Conditions _______________

Grinding Procedure: (attach lab notes)

Sizing Procedure: (attach lab notes)

Solubility Test: Yes ___ No ___ Results ________________________

Primary Stock Preparation:

	gm desired weight		gm sample + tare
+ ________	gm tare	- ________	gm tare after delivery
	gm ideal total		gm test sample

Vortex/Sonication ____

Add F12 medium ____________ ml Lot No. ________________

Time of addition ______ Leach Time _____hr. Conditions ________

Primary stock concentration __________ μg/ml Vol. ____ ml Label A.

Description ____

pH: Normal or ___________________. pH Adjustment: Yes ___ No ___

_____ ml of ______ (Supplier ________ lot # ______) to adjust

_____ ml of primary stock suspension to pH _____.

CHO FORM 6 (Continued)

Assay No. ____________ Date ____________ Tech. ____________

Sponsor ____

2. Aqueous Sample

Sample Identity ________________ Sponsor ID No. ________

Description ____

Warnings/Precautions ___________________________________

Date Received ____________ Storage Conditions ____________

Preparation or Characterization Procedures: (attach lab notes)

Primary Stock Preparation:

Component	Vol. ml	Supplier	Lot No.	Date Thawed	Exp. Date
10X F10					
Serum, heat inactivated					
$NaHCO_3$, 7.5%					
L-Glutamine, 200 mM					
Pen-Strep, 10^4 µg/ml					
Fungizone, 250 µg/ml					
Kanamycin, 10^4 µg/ml					
Deionized water (sterile)					
Test Sample					

Total Volume: ____________ ml

Concentration of primary stock ________ µl/ml, Label A.

Description ____

pH: Normal or ________. pH adjustment: Yes ____ No ____.

____ ml of ____ (Supplier ________ lot # ______) to adjust

____ ml of primary stock to pH ____.

CHO FORM 6 (Continued)

Assay No. ______________ Date ______________ Tech ______________

Sponsor ________________

3. Sample in Organic Vehicle

Sample Identity ____________________ Sponsor ID No. __________

Description ____

Warnings/Precautions ______________________________________

Date Received ______________ Storage Condition ______________

Preparation or Characterization Procedures: (attach lab notes)

Date operations completed _________ Vehicle _________ Volume____ ml
Conc. _________ mg/ml

Prepared by _____________ Storage condition __________________

Description ____

Primary and Diluted Stocks in Organic Vehicle:

Label	Stock Volume, ml	Vehicle Volume, ml	Total Volume, ml	Dilution factor	Sample concentration µl/ml	Sample concentration µg/ml,
I (Primary Stock)						
II						
III						
IV						
V						
VI						
VII						

Vehicle __________ Supplier ____________ Lot No. ____________

Comments ____

CHO FORM 7. PREPARATION OF TEST SAMPLE DOSING STOCKS

Assay No. __________________ Date _____________ Tech. ______________

A. Solid/Particulate/Aqueous Test Samples: Primary stock (A) at ____/ml.

Label	Stock Volume, ml	Medium Volume, ml	Total Volume, ml	Dilution Factor	Sample Concentration µl/ml	µg/ml
B						
C						
D						
E						
F						
G						
H						

B. Test Sample in Organic Solvent:

Label	Stock Volume, ml	Medium Volume, ml	Total Volume, ml	Dilution Factor	Sample Concentration µl/ml	µg/ml
A	of I			50		
B	of I			100		
C	of II			100		
D	of III			100		
E	of IV			100		
F	of V			100		
G	of VI			100		
H	of VII			100		
Vehicle Control				50		
Vehicle Control				100		

Medium ________________ Lot No. _____________ Exp. Date ______
Solvent ________________ Supplier _____________ Lot No. ________

CHO FORM 8. TREATMENT OF TEST CULTURES

Assay No. ___________ Date _______________ Tech ______________

1. Cell Treatment

Treatment started: Time _____________ Vol./dish __________ml

Time elapsed since seeding _________ Treatment sequence _________

Test Medium Label	Plate No.	Sample Conc.	At treatment: Appearance of Dosed Culture	pH	At termination: Appearance of Dosed Culture	pH
A						
B						
C						
D						
E						
F						
G						
H						
NC						
VC						
VC						

NC = Negative Control VC = Vehicle Control (Identify Vehicle Control)

pH meter standardized: Date _____ Init. _____ Date _____ Init. _____

2. Treatment Termination

Date ______ Time _______ Exposure time _____ hr Tech _________

Wash: No. ____ Vol. _____ Type ____ Supplier ________ Lot No. ____

Medium added per dish: Type _______ Volume ______ Lot No. ______

Incubator Identity _________ Conditions ______________________

CHO FORM 9. COLLECTION OF RAW CYTOTOXICITY DATA

Assay No. ____________ Date ______________ Tech __________________

1. Assay Termination

Variations from standard incubator conditions ____________________

Fixative ____________ Stain/Supplier __________________________

Comments ____

2. Colony Counting Date ______________ Tech ____________

COLONY COUNTS

Culture Identity	Plate No.	Applied Conc.	Dish #1	Dish #2	Dish #3	Average Count	Relative Survival %	Cloning Efficiency %
NC								
VC								
VC								
H								
G								
F								
E								
D								
C								
B								
A								

NC = Negative Control
VC = Vehicle Control (Identify Vehicle Control)

Computer Data Storage and Processing: Yes _____ No _______

Entered by ________ Date _____ Program ______ File No. ____

Assay Reviewed by Supervisor ________________ Date __________

CHO FORM 10. CHO CLONAL CYTOTOXICITY DATA REPORTING FORM

Sample Identity ____________________

____

Description of Sample ____________________

Assay No. ____________________

Date Received ____________________

Test Date ____________________

Vehicle ____________________

Cell Type ____________________

Cells Seeded per Dish ____________________

EC_{50} Value ____________________

Toxicity Classification ____________________

pH Alterations ____________________

Comments on Treatment ____________________

____

____

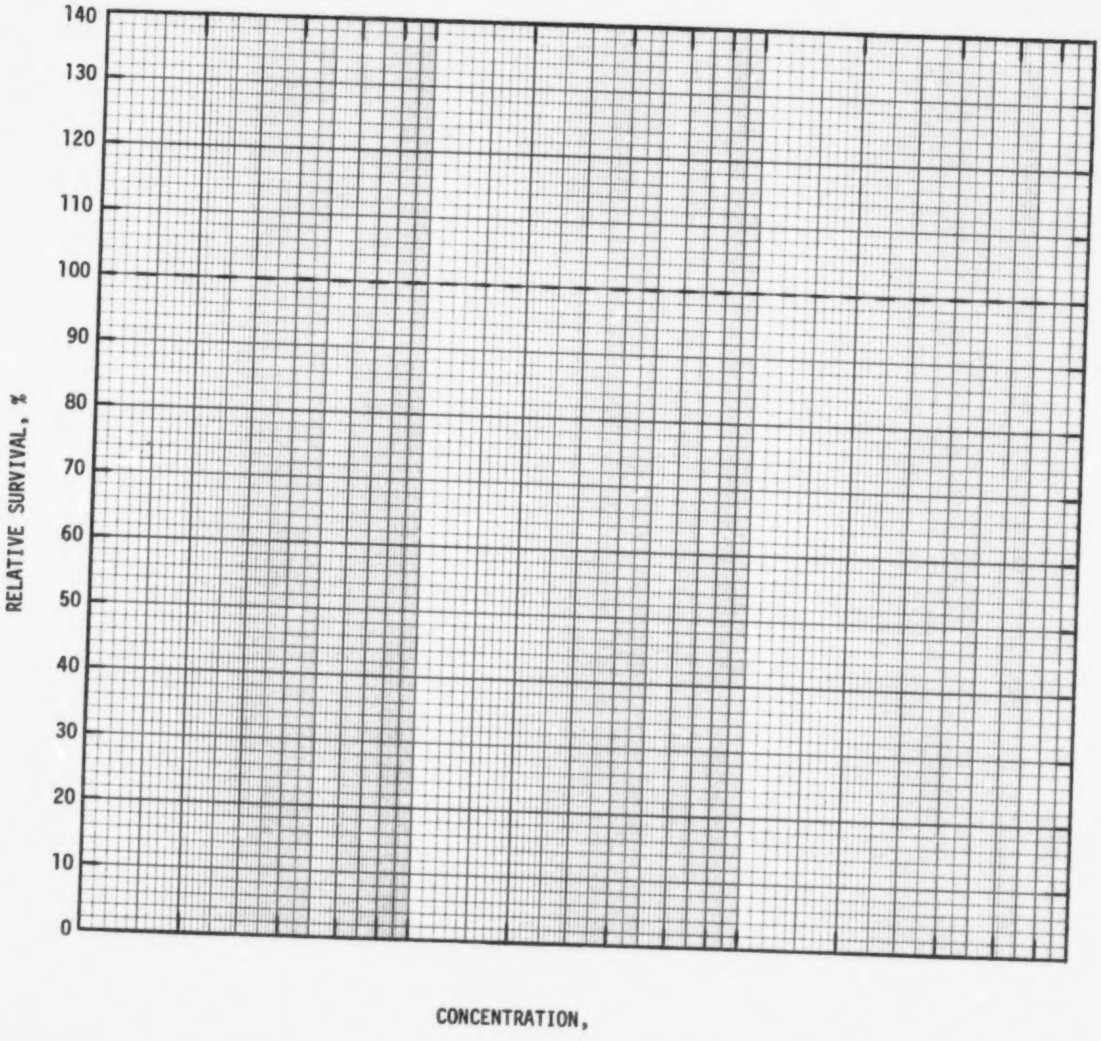
____

COLONY COUNTS

Sample	Applied Concentration ___/ml	Dish #1	Dish #2	Dish #3	Average Count	Relative Survival* %	Cloning Efficiency %
NC							
VC							
VC							
Test							
Test							
Test							
Test							
Test							
Test							
Test							
Test							

NC = Negative Control
VC = Vehicle Control, percent vehicle given
*Relative to NC or appropriate VC

CHO FORM 11. CHO ASSAY EC_{50} DETERMINATION



CHAPTER 6

QUALITY CONTROL PROCEDURES AND DOCUMENTATION REQUIREMENTS FOR THE RABBIT ALVEOLAR MACROPHAGE (RAM) CYTOTOXICITY ASSAY

6.1 INTRODUCTION

The EPA Level 1 Manual defines the scope of the Environmental Assessment Program and provides detailed assay procedures and data analysis for all phases of its implementation. The following sections provide supplementary material specifically related to the quality control and documentation of the Rabbit Alveolar Macrophage (RAM) Cytotoxicity Assay. A uniform approach to the control of assay procedures and the necessary documentation is essential to a meaningful comparison of results obtained in different laboratories.

For convenience in presentation of the various operations, the RAM Cytotoxicity Assay is organized into six steps. A flow diagram of the steps is provided in Figure 4. Step 5 is further subdivided into the two separate analyses that generate the toxicity data. This data is collected and analyzed in Step 6. In the following sections, each step is developed by identifying the separate operations involved, giving the quality control functions associated with the operations, and by defining the necessary documentation. In most cases, the documentation requirements will be satisfied by completely filling out data recording forms similar to those provided at the end of this chapter.

The objective of the RAM assay is to determine the concentration of test material that reduces by 50 percent the number of viable cells and the cellular adenosine triphosphate (ATP) content after a 20-hour exposure. These concentrations are calculated or estimated graphically and are referred to as the EC_{50} values for each measured parameter.

6.2 MACROPHAGE COLLECTION (STEP 1)

6.2.1 Operations

1. A male or female, New Zealand rabbit is sacrificed by injection of sodium pentobarbital (60 mg/kg) into the marginal ear vein.

2. A tracheostomy is performed and normal saline (30 ml) is introduced into the lungs. After 15 minutes, the lavage fluid containing macrophages is removed and placed on ice. Five to nine additional lavages are collected (without a waiting period) and placed on ice.

6.2.2 Quality Control

1. The rabbit is examined before sacrifice to establish clinical health and freedom from obvious respiratory illnesses.

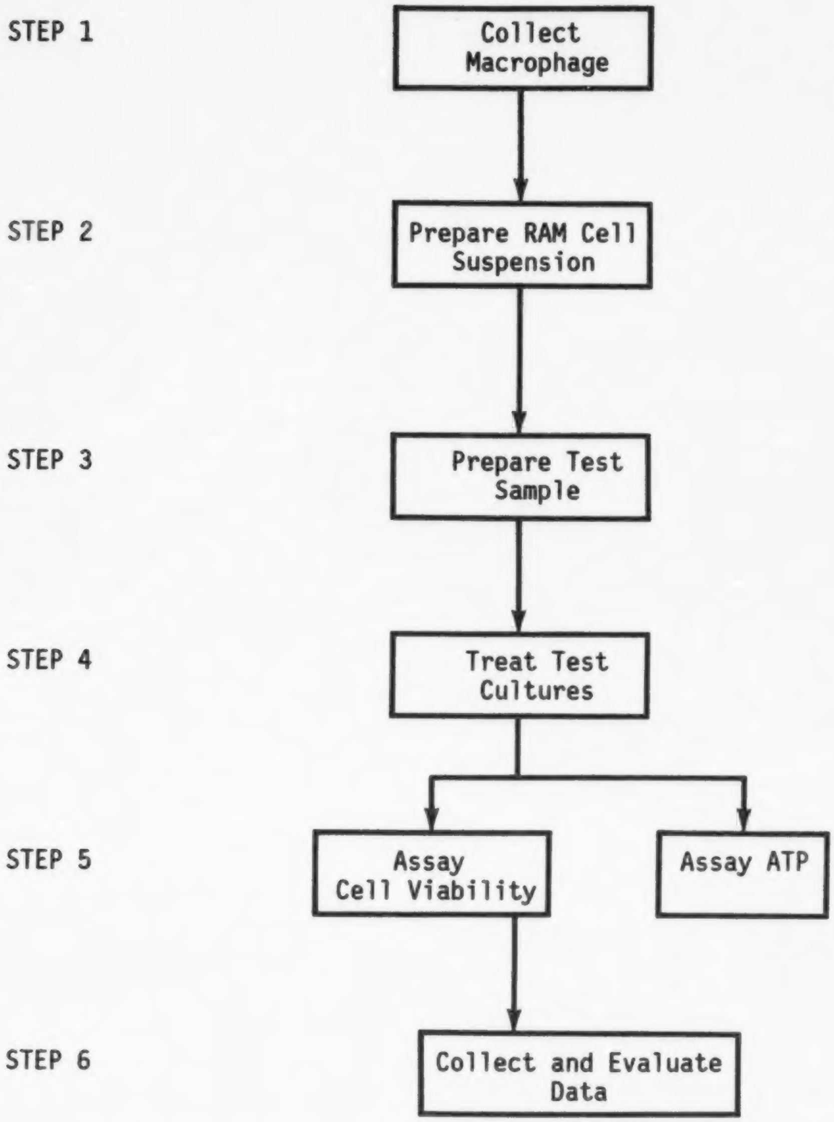

STEP 1
Collect Macrophage
STEP 2
Prepare RAM Cell Suspension
STEP 3
Prepare Test Sample
STEP 4
Treat Test Cultures
STEP 5
Assay Cell Viability
Assay ATP
STEP 6
Collect and Evaluate Data

Figure 4. Rabbit alveolar macrophage (RAM) cytotoxicity assay steps.

2. The operating technician should be trained to perform the tracheostomy procedures and should be familiar with standards for animal studies established by the American Association for Laboratory Animal Science.

3. A healthy rabbit of 1.5 to 2.5 kg should produce more than enough macrophages for one assay. Use of multiple rabbits is desirable for larger-scale testing. Testing and comparison of multiple samples is ideally done with simultaneous treatment using cultures established from a common pool of RAM cells.

4. Sterile technique is used in the tracheostomy and collection of lavages.

5. Any lavage containing significant amounts of mucous or blood is discarded.

6.2.3 Documentation (RAM form 1)

1. The animal source, animal number, identification system, and husbandry conditions are recorded.

2. The operating technician fills out the macrophage collection form (RAM form 1), which becomes a part of the permanent laboratory record for the assay. The form is dated and initialed.

6.3 PREPARATION OF RAM CELL SUSPENSION (STEP 2)

6.3.1 Operations

1. The macrophages are collected from the lavage fluids by centrifugation for 15 minutes at room temperature at 450 x g; the cell pellets are washed once with cold phosphate buffered saline (PBS). The pellets are then resuspended in cold Eagle's Minimum Essential Medium (EMEM) supplemented with 20 percent fetal bovine serum (heat-inactivated for 30 minutes at 56°C) and pooled into one suspension. An alternate culture medium, Medium-199, may be used in place of EMEM. Medium-199 is supplemented in an identical manner as is EMEM.

2. A viable cell count is performed, using a hemacytometer and the trypan-blue dye-exclusion method. The cell suspension is then diluted with medium to between 5×10^5 and 10^6 cells/ml.

3. A differential cell count is obtained from an air-dried smear on a glass slide stained with Wright blood stain.

6.3.2 Quality Control

1. The centrifugation force used in collecting and washing the macrophages should be at, but not exceed, 450 x g for 15 minutes.

2. The viable cell count should be greater than 95 percent. Discard cultures with lower viabilities.

3. A minimum of 200 cells are counted to determine the cell density. If animals yield more than 60 x 10^6 or less than 20 x 10^6 cells, the collection is discarded.

4. The Wright-stained smear is used to verify that macrophages comprise 90 percent or more of the nucleated cell population. Cultures with lower macrophage contents are discarded.

5. Sterile techniques are used in handling the cell suspension. Protection against contamination is provided by routinely supplementing the EMEM with penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (Fungizone) (0.5 µg/ml). Kanamycin may also be added for extra protection against bacterial contamination; normally 35 µg/ml is used, but up to 100 µg/ml may be used.

6.3.3 Documentation (RAM forms 2 through 4)

1. The responsible technician fills out the preparation of cell suspension form (RAM form 2) at the time of the assay. The form is dated and initialed and constitutes part of the permanent laboratory record for the assay.

2. Culture media and reagent logs (RAM form 3) are maintained that identify the quantity, supplier, lot number, and expiration date of each component used.

3. The Wright-stained smear is preserved with Permount and cover slip, indexed and is filed.

4. Daily recordings of temperature, humidity and CO_2 levels are maintained with each incubator and are periodically collected into a permanent record (RAM form 4).

6.4 PREPARATION OF TEST SAMPLE (STEP 3)

6.4.1 Operations

1. Solid samples are ground to homogeneous, fine particles of 5 µm or less or, if supplied as a fine powder, are used without further preparation. The particulate sample is weighed, vortexed dry for two minutes and suspended in EMEM (0 percent FBS) culture medium at 2000 µg/ml. The suspension is rocked at 37°C in a five percent CO_2 humidified atmosphere for 20 hours. Serial dilutions are performed with serum-free EMEM.

2. Aqueous samples and suspensions are used (as supplied) as one of the diluents for a 10x concentrate of EMEM. Normally, 12 ml of sample is used to prepare 20 ml of serum-free test medium at the maximum concentration of 600 µl/ml. Serial dilutions are performed with serum-free EMEM.

3. Samples supplied in an organic solvent, are solvent exchanged to dimethylsulfoxide (DMSO) and are added to serum-free EMEM at twice the final concentration. The highest test concentration represents a 1:25 dilution of sample to four percent of medium volume. Lower concentrations are prepared by serial dilution in DMSO yielding two percent solvent. Normally, 1.4 ml of the test sample is consumed per assay.

4. Additional operations, such as grinding to a specified upper size limit, extractions, concentrations, and solvent-exchange into DMSO may be specified for test samples. The final form will conform to one of the above descriptions.

6.4.2 Quality Control

1. Upon receipt, each sample is given a unique identification number, is described physically, and is stored as specified (or as appears appropriate).

2. All chemical and physical transformations of the sample are described in a manner that allows the final preparation to be related in quantity and/or concentration to the originally supplied sample.

3. Fine, particulate materials are placed in EMEM (0 percent FBS) to leach for 20 hours at 37°C with rocking.

4. Adequate protection against microbial contamination introduced by the test sample is normally provided by penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.5 μg/ml) in the test media. Kanamycin (35 to 100 μg/ml) may also be added for extra protection against bacterial contamination.

5. A description of the test sample distribution in and reaction with the test media must be provided. Any pH changes outside the normal culture range are measured.

6. The pH meter is calibrated with reference pH solutions before use.

7. If sample causes a drop in pH below 6.8 or an increase above 7.6, the sample is retested with the pH adjusted to pH 7.2. The pH may be adjusted to pH 7.2 using ultrapure HCl or NaOH.

6.4.3 Documentation (Ram forms 5 and 6)

1. The responsible technician fills out the following forms (RAM forms 5 and 6) for the sample type at the time of the assay. Each form is dated and initiated and constitutes a part of the permanent laboratory record of the assay.

2. Records from the responsible unit doing test sample manipulations are obtained and maintained with the bioassay data record.

3. Culture media, reagent and incubator logs are maintained. See Section 6.3.

4. Records are obtained and maintained documenting removal of aliquots of test material from the chemical repository, the use of the analytical balance and the pH meter.

6.5 TREATMENT OF TEST CULTURES (STEP 4)

6.5.1 Operations

1. Serum-free EMEM containing the test sample or solvent at twice the desired final concentration (previously prepared in Section 6.4) is distributed into 25-cm^2 flasks that have been pregassed for at least two hours in a humidified atmosphere containing 5 percent CO_2. Each flask receives 2.0 ml aliquots of the test sample dilution; three flasks are prepared for each test concentration and for the appropriate negative and vehicle controls.

2. Flasks are inoculated with cells by adding to each flask 2.0 ml aliquots of the 5×10^5 to 1×10^6 cells per ml cell suspension in EMEM with 20 percent FBS (previously prepared in Section 6.3).

3. The dosed and inoculated flasks contain the test material at the desired final concentration, between 1×10^6 and 2×10^6 cells per flask and 4 ml of EMEM at 10 percent FBS.

4. The flasks are incubated at 37°C with five percent CO_2 and saturated humidity on a stationary rocker platform for about 30 minutes. Then the flasks are slowly rocked (12 oscillations per minute) for the remainder of a 20-hour exposure period.

5. Alternate dosing configurations may be used. Six-well cluster dishes may be used in place of 25-cm^2 flasks. If cluster dishes are used, each well is inoculated with 1 ml of the test material in EMEM (0 percent FBS) at twice the desired final concentration and 1 ml of cell suspension in EMEM (10 percent FBS) at 1×10^6 to 2×10^6 cells per ml.

6.5.2 Quality Control

1. The macrophages are introduced into the test media within two hours from collecting the first lung lavage. Longer time delays can be avoided by diluting the test media and preparing the test flasks while the cells are being washed with saline.

2. Three cultures are exposed to each test concentration to allow for contamination losses and to determine average responses.

3. Equal numbers of cells are placed in each test culture flask. This procedure requires accurate pipeting from a cell suspension that is swirled frequently to maintain a homogeneous distribution of cells. Cells may also be prepared from a spinner flask to ensure a homogeneous cell suspension.

4. The exposure conditions are standardized at 4 ml of test medium at 37°C for 20 hours to help ensure uniform results between laboratories. The maximum applied concentrations are standardized at 1000 µg/ml for particulate materials, 600 µl/ml for aqueous samples, and 20 µl/ml for test samples supplied in DMSO.

5. Changes in the pH from the normal culture range are recorded because this factor may be responsible for the observed toxicity. If the sample causes a drop in pH below 6.8 or an increase above 7.6, the sample is retested with the pH adjusted to 7.2.

6. The pH meter is calibrated with reference pH solutions before use.

6.5.3 Documentation (RAM form 7)

1. The responsible technician fills out the appropriate form (RAM form 7) at the time of operations. The form is dated and initialed and constitutes part of the permanent laboratory record for the assay.

2. Culture media, reagent, incubator and pH meter logs are maintained. See Section 6.3.

6.6 CELL VIABILITY ASSAY (STEP 5A)

6.6.1 Operations

1. At the end of the treatment period, the medium containing unattached cells is transferred with a pipet into a centrifuge tube on ice. The attached cells are rinsed with 1 ml of freshly thawed 0.1 percent trypsin/0.01 percent EDTA and then incubated with 2 ml of the trypsin/EDTA solution for about 5 minutes at 37°C. The wash, typsinate, and medium are combined for each culture to yield a 7 ml suspension that is held on ice.

2. A 1.0 ml aliquot is removed for cell count and viability determination, using a hemocytometer and the trypan-blue dye-exclusion method.

3. If cluster dished are used, cells are harvested in a similar manner as with 25-cm^2 flasks. The culture medium is removed by pipet to centrifuge tube on ice and the cells detached by adding 1 ml of the trypsin/EDTA solution for five minutes. The trypsinates and medium are combined for each culture to yield a 3 ml suspension. Aliquots of 0.5 ml are taken for cell counts.

4. Samples of each cell suspension are also removed for ATP assay (Step 5B).

5. The number of live, dead and total cells are recorded manually, or if a computer is used for data storage and processing, entered into the computer. The number of live and total cells per flask is calculated as described in RAM form 8.

6.6.2 Quality Control

1. Changes in the pH from the normal culture range are recorded at the end of the treatment period because this factor may be responsible for observed toxicity.

2. The pH meter is calibrated with reference pH solution before use.

3. The trypsinization time should stay within 5 to 10 minutes to avoid a loss of viability from procedural causes.

4. A visual check of the culture-flask surfaces should be performed to confirm the release of all cells into the trypsinate.

5. Each cell suspension is mixed to ensure a homogenous distribution of cells prior to removal of the 1.0 ml aliquot for counting.

6. Cell suspensions contain test material so caution should be used when handling, counting and disposing of such suspensions.

7. Final data tables are proofed against raw data collected on RAM form 8.

6.6.3 Documentation (RAM form 8)

1. The responsible technician fills out the appropriate form (RAM form 8) at the time of operations. The forms are dated and initialed and constitute a part of the permanent laboratory record for the assay.

2. The reagent and pH meter logs are maintained. See Section 6.3.

3. If additional technicians perform cell counts for some of the doses, they must initial the appropriate comments space.

4. Data entry into the computer and the file name of the data set are documented.

5. If flasks are missing from a dose-set, the reason for the loss is identified; contamination (C), technical error (T), or flask not set up to conserve sample (S).

6. The laboratory supervisor reviews and initials the viability data.

6.7 ADENOSINE TRIPHOSPHATE (ATP) ASSAY (STEP 5B)

6.7.1 Operations

1. ATP is analyzed by extraction of a 0.1 ml cell suspension sample with 0.9 ml of 90 percent DMSO/10 percent 0.01 M MOPS (morpholino-propane sulfonic acid). The mixture is vortexed for ten seconds and after sitting for the remainder of a two minute extraction period at room temperature, 5.0 ml cold 0.01 M MOPS buffer at pH 7.4 is added. The extract is mixed well and placed on ice.

2. A 10 µl aliquot of the extract is injected into a cuvette at room temperature containing a luciferin-luciferase reaction mixture. The cuvette is located in the light-tight chamber of a DuPont Model 760 Luminescence Biometer. A direct read-out of the ATP content is obtained.

3. ATP concentrations are recorded manually, or if a computer is used for data storage and processing, entered into the computer. From these values, parameters based upon ATP content are calculated as described in RAM form 9.

6.7.2 Quality Control

1. Each cell suspension is mixed to ensure a homogeneous distribution of cells prior to removal of the 0.1 ml sample.

2. The cell suspensions are extracted and analyzed as soon as possible. The suspensions and extracts must be kept on ice and the extracts should be analyzed within one hour of preparation. The cells or extracts should not be frozen for ATP analysis at a later date.

3. The Biometer is calibrated just prior to use with standard ATP solutions so that a direct read-out of the ATP content can be obtained. The calibration procedures given in the Biometer manual (1) are followed.

4. Each extract is assayed at least twice to demonstrate consistent readings. The average reading is used for subsequent calculations.

5. Final data tables are proofed against raw data collected on RAM form 9.

6.7.3 Documentation (RAM forms 9 and 10)

1. The responsible technician fills out the appropriate form (RAM form 9) at the time of operations. The forms are dated and initialed and constitute a part of the permanent laboratory record for the assay. If calcuations are done at a later time, the work should be initialled and dated.

2. A reagent log is maintained that identifies the quantity, supplier, lot number, and expiration date of each component used. See Section 6.3.

3. A maintenance and use record on the Biometer must be kept (RAM form 10).

4. Data entry into the computer and the file name of the data set are documented.

5. If flasks are missing from a dose-set, the reason for the loss is identified; contamination (C), technical error (T), or flask not set up to conserve sample (S).

6. The laboratory supervisor reviews and initials the ATP assay data.

6.8 COLLECTION AND EVALUATION OF DATA (STEP 6) (RAM FORMS 11 THROUGH 13)

The viability and ATP assay data are collected and reported on a Standard Form such as that provided in this Section. Absolute values for the assay parameters should be provided as well as the effect of the test material relative to the negative control or appropriate vehicle controls. In addition, the relative data for each parameter is presented to graphically show the determination of the EC_{50} values.

6.8.1 Acceptance Criteria

To consider the assay results acceptable for an evaluation of the test material toxicity, the assay must have been performed within the guidelines given by the Quality Control items in the previous Sections. The final results must also meet the following criteria:

A. The survival of viable macrophages in the negative control cultures over the 20 hour treatment period is 70 percent or greater.

B. The ATP content of the negative control cell culture is between 17 and 37 x 10^8 femtograms ATP per 10^6 total cells.

C. The data points critical to the location of the EC_{50} for the most sensitive assay parameter are the averages of at least two treated cultures.

D. A sufficient number of test concentrations are available to clearly locate the EC_{50} of the most sensitive assay parameter within a toxicity region defined under Toxicity Evaluation Criteria.

6.8.2 Toxicity Evaluation Criteria

The EC_{50} value represents the concentration of test material that reduces an assay parameter to 50 percent of the negative control or appropriate solvent control value. Toxicity data can be analyzed using statistical methods such as those developed by Garrett and Stack (2). Four statistical programs are available for a programmable calculator with magnetic-card capability. Data generated by the programs include the mean and standard deviation of replicate values, probability (P) values, the estimated dose level at which a response attains a given magnitude (normally the EC_{50}), and the 95 percent confidence limits for a given EC_{50} determination. An alternate method to estimate EC_{50} values is to graphically fit a curve by eye through the data points associated with each test parameter plotted as a function of the logarithm of the applied concentration. Each point normally represents the average of three culture flasks for each treatment.

The evaluation of the test material is based upon determination of the EC_{50} values for the four parameters: percent viability (ratio of viable cells to total cells x 100 percent for each treatment), viability index (ratio of viable cells for each treatment to viable cells in the negative control x 100 percent), total ATP content, and ATP per 10^6 cells. The

ATP content will generally be a more sensitive parameter than ATP per 10^6 cells because any cell loss due to treatment will increase the latter parameter. ATP released into the growth medium by disrupted cells contributes to the ATP measurement.

The toxicity of the test material is evaluated as high, moderate, low, or nondetectable according to the ranges of EC_{50} values defined in Table 14. In the case of organic extracts, the evaluation is based upon the weight (μg) of the extract per ml of culture medium. The assay parameter yielding the lowest EC_{50} will classify the test material. The EC_{50} of sorbent extracts of SASS train samples is also converted to the equivalent volume of gas originally sampled (in dry standard cubic liters or feet).

TABLE 14. RAM ASSAY EVALUATION CRITERIA

Toxicity	Solids (EC_{50} in μg/ml)	Aqueous Liquids (EC_{50} in μl/ml)	Nonaqueous Liquids[a] (EC_{50} in μl/ml)
High	<10	<6	<0.2
Moderate	10 to 100	6 to 60	0.2-2
Low	100 to 1000	60 to 600	2-20
Not Detectable	>1000	>600	>20

[a] Criteria for nonaqueous liquids are tentative and under evaluation.

6.9 REFERENCES

1. E.I. DuPont De Nemours and Co., Inc., Instrument Product Division. Instruction Manual for the DuPont 760 Luminescence Biometer, Wilmington, DE 19898.

2. Garrett, N.E. and H.F. Stack. Cellular Toxicology Data Analysis Using a Programmable Calculator. Comput. Biol. Med., 10(3):153-167, 1980.

6.10 SAMPLE FORMS FOR THE RAM CYTOTOXICITY ASSAY

The following set of data recording and standard procedure documentation forms are adequate to provide the necessary information to audit the EPA Level 1 rabbit alveolar macrophage (RAM) cytotoxicity assay.

RAM FORM 1. MACROPHAGE COLLECTION

Assay No. ______________ Date ______________ Tech. ______________

Sponsor ____

1. Rabbit Information

Animal ID No. _______ ID System _______ Date Received _________

Strain ____________________ Sex ____ Weight at Use ___________

Supplier _________ P.O. # _________ Quarantine ______________

Diet/Holding Conditions ______________________________________

Rabbit Health at Use ____

Animal Certified for Use by ____________________ Date ________

Na pentobarbital: Conc. ____________ Vol. __________________

Source ___________ Lot No. ___________ Exp. Date ________

2. Macrophage Collection

RAM Cell Culture No. _________________

First lavage volume __________ Time In _______ Time Out ______

Observations ____

No. of additional lavages ____________ Vol./lavage ____________

0.85% Saline (PBS): Supplier ___________ Lot No. _____________

Time when collections completed _____________________________

Comments on tracheostomy:

Comments on lavages:

RAM FORM 2. PREPARATION OF CELL SUSPENSION

Assay No. ______________ Date ______________ Tech. ______________

1. Cell Washing:

Centrifuge ID ________ Head ______ RPM _______ G's ________

Centrifugation 1: Time ____ to ____ Resuspend _____ ml PBS/tube

Centrifugation 2: Time ___ to ___ Resuspend __ ml EMEM/tube

Pooled cell suspension vol. ____ ml. EMEM w/20% FBS Lot No. ____

PBS: Supplier _______________ Lot No. ______________

2. Cell Count and Dilution:

Dilution: __ ml cells + __ ml trypan blue, Dil. factor ______

Count in ten 10^{-4} ml areas:

Count 1 ____ Live ____ Dead ____ Total

Count 2 ____ Live ____ Dead ____ Total

Average: ____ Live ____ Dead ____ Total

% Viability = (Live count/Total count) x 100 ________ = _______

Cell density = Live count x dil. factor x 10^3 ________ = _______

Comments ____

Trypan blue supplier __________ Conc. __________Lot No. ________

Calculations:

Dilution: ____ ml cells + __ ml EMEM w/20% FBS Lot No. ____

Final Cell density = ___________ cells/ml Volume __________ ml

(Deliver 2 ml per flask, yielding ____ x 10^6 viable cells seeded)

3. Wright-Stain Cell Count: Date __________ Tech. __________

Total Macrophage _______________ Total Other Cells ____________

Total Cell Count _______________ % Macrophage ________________

Wright Stain: Supplier __________________ Lot No. __________

RAM FORM 3. MEDIA AND REAGENT LOG

Product ____________________ Date ______________

Assigned Lot No. ______________ Tech ______________

Expiration Date ______________

Component	Amount	Supplier	Lot No.	Exp. Date

RAM FORM 4. INCUBATOR LOG

I.D. No. _______

Chamber _______

Date	Time	Gauge Readings CO_2/Air	CO_2 Level %	CO_2 Pressure Line/Tank	Humidity	Temp °C	Changes	Tech Initials

RAM FORM 5. PREPARATION OF PRIMARY TEST SAMPLE STOCK

Assay No. ____________________ Date ______________ Tech. _______________

Sponsor ____

1. Solid/Particulate Sample:

Sample Identity ______________________ Sponsor ID __________________

Description ____

Warnings/Precautions ____

Date Received __________________ Storage Condition ________________

Grinding Procedure: (attach lab notes)

Sizing Procedure: (attach lab notes)

Primary Stock Preparation:

gm desired weight — gm sample + tare

+ ________ gm tare — - ________ gm tare after delivery

gm ideal total — gm test sample

Add serum-free EMEM _____ ml Lot No. _____________ Exp. Date _____

Solubility ____

Time of addition ______________________ Sonication _________________

Time of Addition ______________ Leach Time ____________hr

Conditions ____________________ Vortex/Sonication _________________

Primary 2x stock concentration _______μg/ml Vol. ______ml Label A.

Description ____

pH: Normal or ___________________. pH adjustment: Yes _ No ___

_____ ml of _____ (Supplier ____________, Lot # ____)to adjust

____ ml of primary stock to pH _____.

RAM FORM 5 (Continued)

Assay No. ____________________ Date ______________ Tech. _______________

Sponsor ____

2. Aqueous Sample:

Sample Identity ____________________ Sponsor ID No. _______________

Description ____

Warnings/Precautions ____

Date Received __________________ Storage Condition ________________

Preparation or Characterization Procedures: (attach lab notes)

Primary Stock Preparation:

Component	Volume, ml	Supplier	Lot No.	Date Thawed	Exp. Date
10X EMEM					
$NaHCO_3$, 7.5%					
L-glutamine, 200 mM					
Pen-Strep, 10^4 µg/ml					
Fungizone, 250 µg/ml					
Kanamycin, 10^4 µg/ml					
Deionized water					
Test Sample					

Total Volume: __________ ml

Concentration of Primary 2x Stock __________µl/ml Label A.

Description ____

pH: Normal or ______________________. Adjustment: Yes ___ No ___

_____ ml of _____ (Supplier _______________, Lot # _____) to

adjust _____ ml of primary stock to pH _____.

RAM FORM 5 (Continued)

Assay No. ______________ Date ____________ Tech. ____________

Sponsor ____

3. Sample in Organic Vehicle:

Sample Identity ______________ Sponsor ID No. ____________

Description ____

Precautions/Warnings ________________________________

Date Received ______________ Storage Condition ____________

Preparation or Characterization Procedures of Primary Stock: (attach lab notes)

Date operations completed _____. DMSO Vol. ____ ml Conc. ____ mg/ml

Prepared by ________________ Storage Condition ____________

Description ____

Dilution of Primary Stock in Organic Vehicle:

Label	Stock Volume, ml	DMSO Volume, ml	Total Volume, ml	Dilution Factor	Sample Concentration µl/ml	Sample Concentration µg/ml
I Primary Stock						
II						
III						
IV						
V						
VI						
VII						

Vehicle __________ Supplier ______________ Lot No. __________

Comments ____

RAM FORM 6. PREPARATION OF 2X TEST SAMPLE STOCKS

Assay No. ____________________ Date ______________ Tech. _______________

A. Solid/Particulate/Aqueous Test Samples: Primary stock (A) at _____/ml.

Label	Stock Volume, ml	Medium Volume, ml	Total Volume, ml	Dilution Factor	Sample Concentration µl/ml	µg/ml
B						
C						
D						
E						
F						
G						
H						

B. Test Sample in Organic Solvent:

Label	Stock Volume, ml	Medium Volume, ml	Total Volume, ml	Dilution Factor	Sample Concentration µl/ml	µg/ml
A	of I			25		
B	of I			50		
C	of II			50		
D	of III			50		
E	of IV			50		
F	of V			50		
G	of VI			50		
H	of VII			50		
Vehicle Control				25		
Vehicle Control				50		

Serum-Free EMEM Lot No. ______________________ Exp. Date ____________

Solvent ___________ Supplier____________________ Lot No. ______________

RAM FORM 7. TREATMENT OF TEST CULTURES

Assay No. ________________ Date _____________ Tech. __________

1. Cell Treatment:

Vol. of test medium/flask ___ ml Flask Size ____ No. of Flasks ____

Vol. of cell suspension/flask ___ ml Cells seeded/flask _________

Time of treatment ____________ Treatment sequence ____________

Incubator Identity ____________ Conditions _________________

			At treatment:		At termination:	
Test Medium Label	Flask No.	Sample Conc.	Appearance of Dosed Culture	pH	Appearance of Dosed Culture	pH
A						
B						
C						
D						
E						
F						
G						
H						
NC						
VC						
VC						

NC = Ngative Control VC = Vehicle Control (Identify Vehicle Control)

pH metr standardized: Date _____ Tech. _____

2. Tratment Termination

Dte ______ Time _______ Exposure time _____ hr Tech ________

(see Cel Viability Assay for cell collection data)

RAM FORM 8. CELL VIABILITY ASSAY

Assay No. ______________ Date ______________ Tech. ______________

1. Harvest Data:

0.1% Trypsin/0.01% EDTA Lot No. ________ Exp. Date ____________

Total Suspension Vol. _____ ml Exceptions________________________

2. Cell Count and Viability:

Dilution: ___ ml suspension + ___ ml trypan blue; Dil. Factor _____

Trypan blue supplier _________ Conc. _________ Lot No. ________

Dose & Flask Iden.	Cell Count			Cells per flask x 10^6		% Viability	Viability % Control	Viability Index	Comments
	Live	Dead	Total	Live	Total				
A									
B									
C									
AVE									
A									
B									
C									
AVE									
A									
B									
C									
AVE									
A									
B									
C									
AVE									
A									
B									
C									
AVE									

RAM FORM 8 (Continued)

Assay No. ______________ Date ______________ Tech. ______________

2. Cell Count and Viability (Continued):

Dose & Flask	Cell Count			Cells per flask x 10^6					
Iden.	Live	Dead	Total	Live	Total	% Viability	Viability % Control	Viability Index	Comments
A									
B									
C									
AVE									
A									
B									
C									
AVE									
A									
B									
C									
AVE									
A									
B									
C									
AVE									
A									
B									
C									
AVE									
A									
B									
C									
AVE									

Computer Data Storage and Processing: Yes ____ No ____

Entered by _______ Date _______ Program Name ________ File No. _____

Reviewed by Supervisor ____________________ Date ____________________

RAM FORM 9. ATP ASSAY

Assay No. ________________ Date ______________ Tech. ______________

1. Luciferin-Luciferase Reaction Mix (RM) Preparation:

Buffer salt: ________ tablets Source ________ Lot No. ________

Reaction mix: ________ vials Source ________ Lot No. ________

Low resistance water: ___ ml Source ________ Lot No. ________

Dissolve buffer salt tables in low resistance water, then dissolve reaction mix (enzyme and substrate), mix gently. Time ____________

2. 90% DMSO Preparation:

90% DMSO: ______ ml DMSO + _____ ml 0.01M MOPS [Lot No. ________]

DMSO Source ____________________ Lot No. ____________________

3. Biometer Calibration: Refer to Instrument Manual.

A. Reaction mix: Inherent light and dark current for RM ________

- Dark current ________

Inherent light ________

B. MOPS buffer: Injection of 10 μl MOPS buffer ________

Should be less than 1.0 with no exponent change.

C. Calibration with 1 x 10^8 fg ATP/ml standard:

ATP Standard: Lot. No. _________ Exp. Date __________

Initial ATP Readings		ATP Readings After Adjustment	
________________		________________	
________________		________________	
________________	________ mean light response	________________	________ mean light response
		________________	

Adjust sensitivity to 1.0 x 10^8 fg ATP/ml

Calibration completed: Tech. ________ Date ________ Time ________

Note: Femtogram (fg) is equal to 10^{-15} gram.

Comments:

RAM FORM 9 (Continued)

Assay No. ______________ Date ____________ Tech. _____________

4. ATP Extraction and Assay:

Extraction: ______ to _______ ATP Assay: ________ to ________

____ ml cells, _____ ml 90% DMSO, _____ ml MOPS; Dil. Fact. ____

Dose & Flask Iden.	Biometer Readings	Flask Average	Dose Average	fg ATP per Flask (Ave.xD.F.)	Flask % Control	fg ATP per 10^6 Total Cell	ATP/ 10^6 Cells % Control
A							
B							
C							
A							
B							
C							
A							
B							
C							
A							
B							
C							
A							
B							
C							
A							
B							
C							

RAM FORM 9 (Continued)

Assay No. ________________ Date ______________ Tech. _______________

4. ATP Extraction and Assay: (Continued)

Dose & Flask Iden.	Biometer Readings	Flask Average	Dose Average	fg ATP per Flask (Ave.xD.F.)	Flask % Control	fg ATP per 10^6 Total Cell	ATP/ 10^6 Cells % Control
A							
B							
C							
A							
B							
C							
A							
B							
C							
A							
B							
C							
A							
B							
C							
A							
B							
C							

Computer Data Storage and Processing: Yes _________ No _________

Entered by ________ Date ________ Program Name ________ File No. _______

Reviewed by Supervisor ____________________________ Date ______________________

RAM FORM 10. LUMINESCENCE BIOMETER LOG

DUPONT MODEL 760

DATE	TECHNICIAN INITIALS	USE OR MAINTENANCE OPERATIONS

RAM FORM 11. RAM CYTOTOXICITY DATA FORM

Assay No.:

Sample Identity:

Sponsor ID No.:

Test Date:

Vehicle:

Initial Cell Viability:

Viable Macrophage Seeded/Flask:

Macrophage Population Percentage:

Survival of Negative Control Macrophage Over Treatment Time:

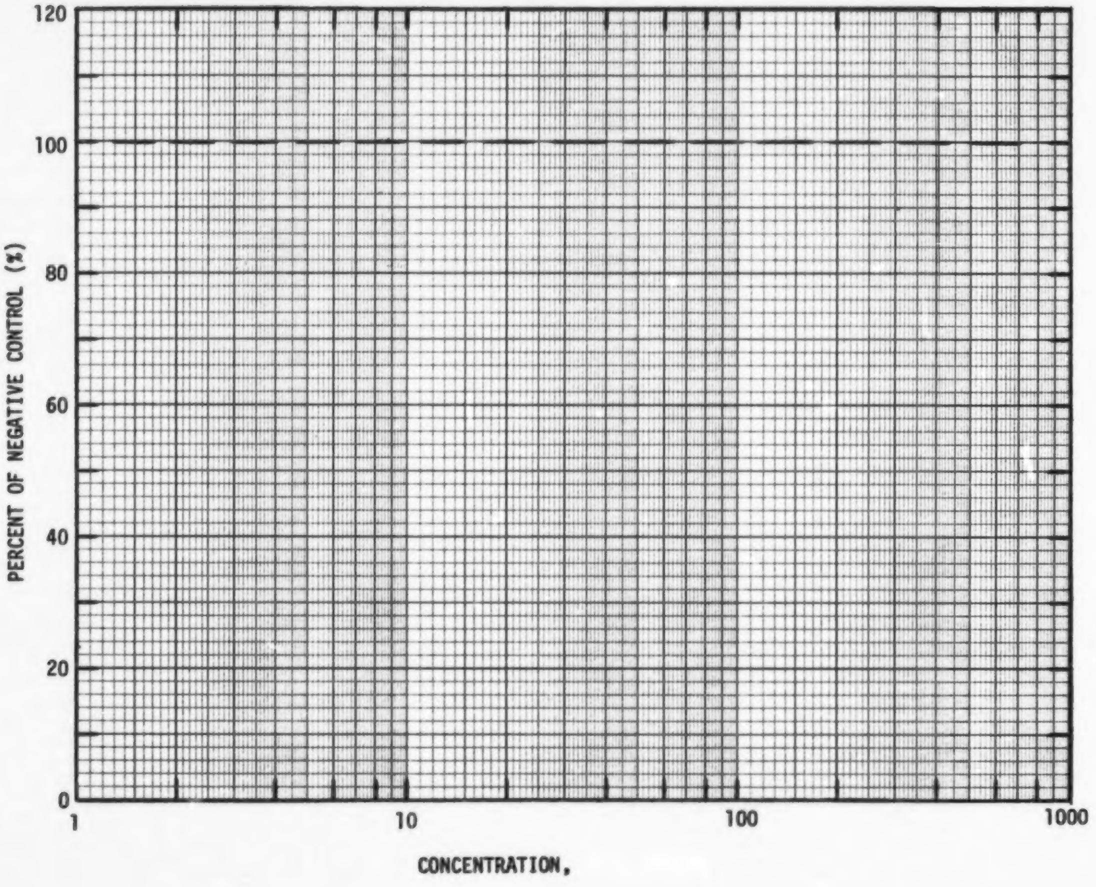
		Average Values per Culture Flask					Expressed as Percent of Control			
Sample	Concentration[a] /ml	Viable Cells 10^6 Units	Total Cells 10^6 Units	ATP 10^8 fg[b]	ATP Per 10^6 Cells 10^8 fg	Viability %	Viability	Viability Index	ATP	ATP Per 10^6 Cells
NC[c]										
VC[d]										
VC										
TEST										
TEST										
TEST										
TEST										
TEST										
TEST										
TEST										

EC50 VALUES:
μg/ml: ____________________

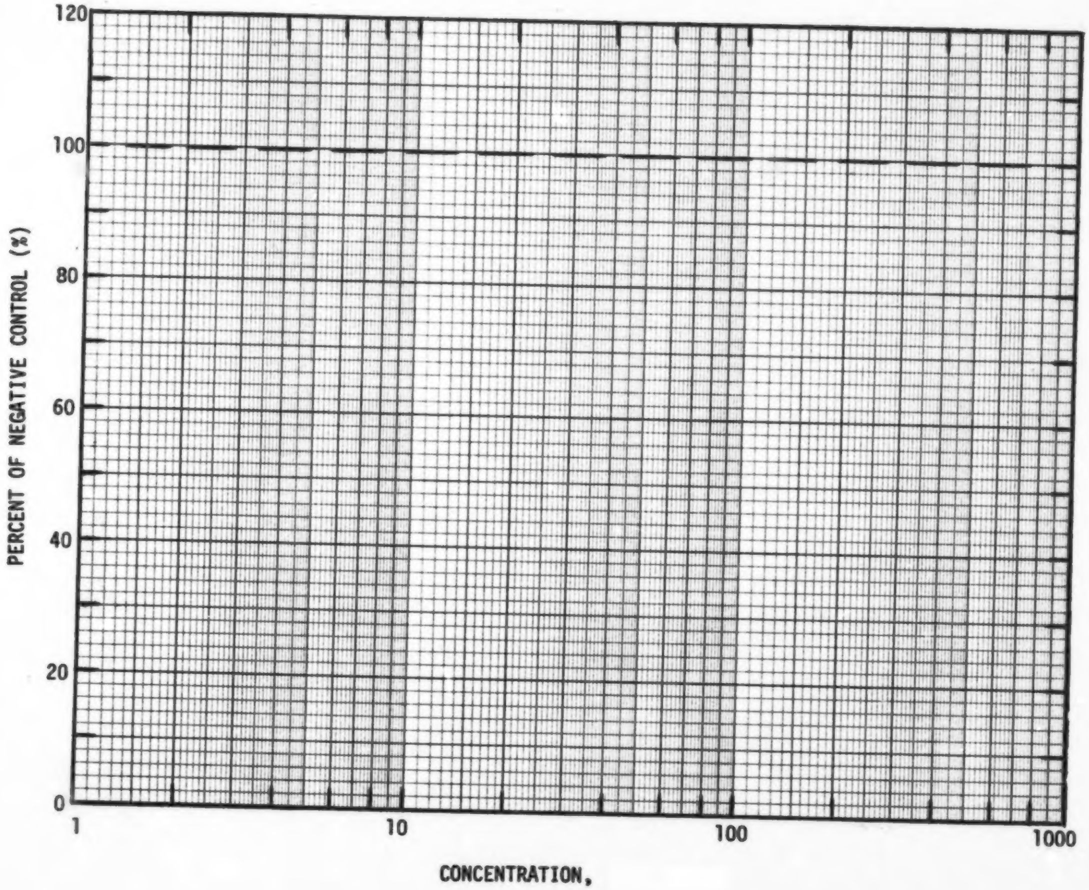
Toxicity Classification:

[a] pH change in culture medium:
[b] fg = femtogram (10^{-15} gram).
[c] NC = Negative Control
[d] VC = Vehicle Control

RAM FORM 12. RAM ASSAY EC_{50} DETERMINATION (VIABILITY)



RAM FORM 13. RAM ASSAY EC_{50} DETERMINATION (ATP)



CHAPTER 7

QUALITY CONTROL PROCEDURES AND DOCUMENTATION REQUIREMENTS FOR THE IN VIVO RODENT TOXICITY (WAT) ASSAY

7.1 INTRODUCTION

The EPA Level 1 procedures manual defines the scope of the Environmental Assessment program and provides detailed study designs and data analysis for all phases of its implementation.

The following sections describe the quality control and study documentation steps proposed for the EPA Level 1 *in vivo* rodent toxicity assay (WAT) using weanling mice. The steps of the toxicity study are outlined in the flow diagram shown in Figure 5. In most cases, the documentation requirements will be satisfied by completing assay documentation and data recording forms included for reference at the end of this chapter.

The objective of the *in vivo* rodent toxicity assay is to determine the concentration of test material that is lethal to 50 percent of the test animals (LD_{50}). In addition to mortality, physiological observations are recorded on the response of test animals to the test material. This assay is designed in two stages; the first is based upon the quantal (all-or-none) response and the second upon the quantitative (graded) response. Normally, the quantal test (using only the maximum applicable dose of 5 gm/kg or 5 ml/kg) is used to determine the necessity for carrying out the more extensive multiple-dose-level quantitative assay. If no animals die in the quantal phase of testing, no further testing is necessary, and the sample is ranked as having nondetectable toxicity.

7.2 ANIMAL CARE (STEP 1)

7.2.1 Operations

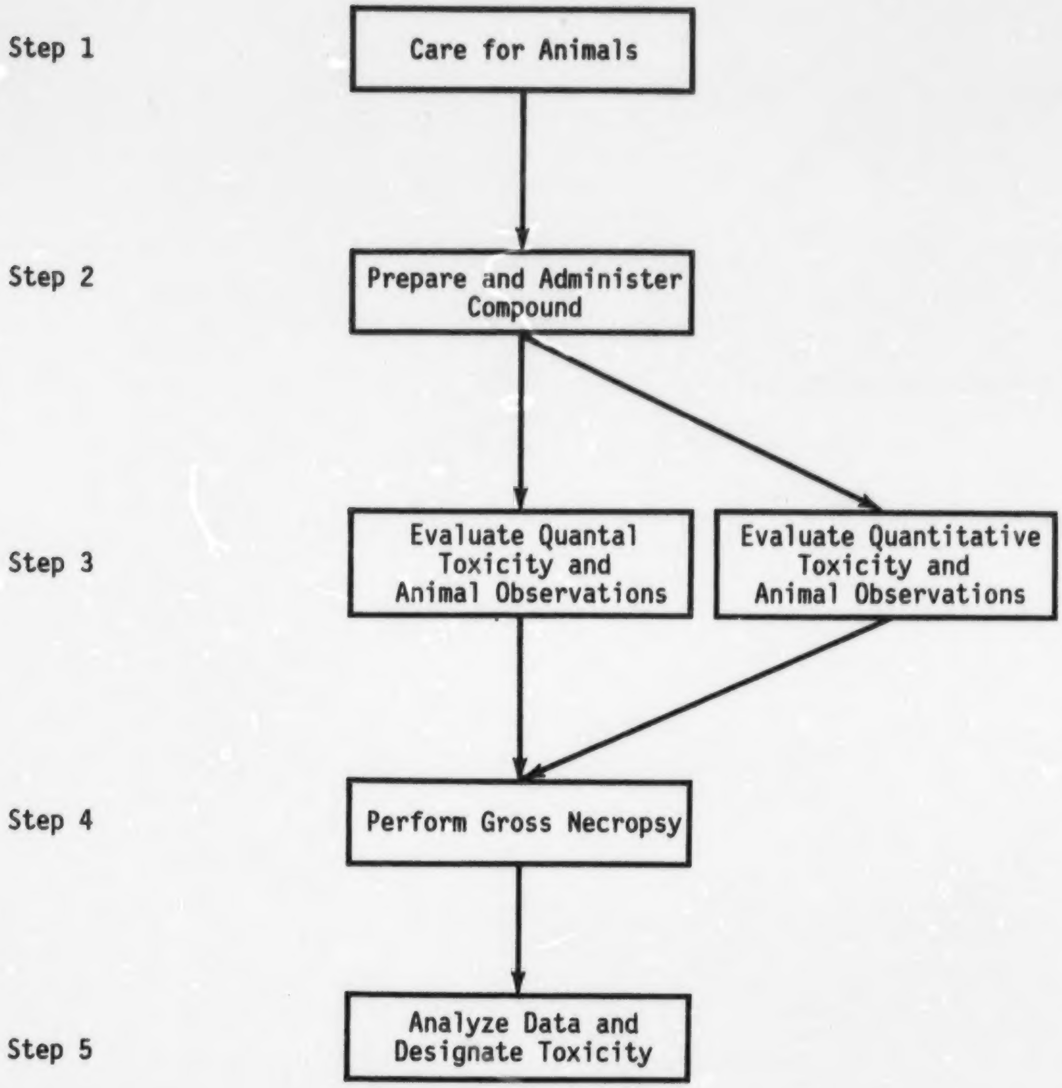
1. Weanling mice (strain CD-1 or equivalent) are obtained from a suitable source. Mice may be bred in laboratory colonies or purchased commercially. If purchased commercially, females with timed pregnancies or mothers with nursing young should be obtained.

2. The animals are placed in quarantine upon entering the facility and remain there for a minimum of 5 days. The mice are observed daily from the date they enter the facility until they are sacrificed.

3. The animals are individually weighed prior to going on study to ensure the proper weight range and are individually ear tagged for permanent identification.

4. Purina Certified Lab Chow and acidified water (pH 2.5) are provided *ad libitum* unless contraindicated by the study director.

5. Animals are released to go on study following quarantine and a physical examination.


Step 1
Care for Animals
Step 2
Prepare and Administer Compound
Step 3
Evaluate Quantal Toxicity and Animal Observations
Evaluate Quantitative Toxicity and Animal Observations
Step 4
Perform Gross Necropsy
Step 5
Analyze Data and Designate Toxicity

The Rodent Toxicity Test is divided into five steps as outlined above. The procedures and quality control functions needed to adequately perform these steps are described in the following sections.

Figure 5. *In vivo* rodent toxicity assay steps.

7.2.2 Quality Control

1. Litters should be adjusted to five male and five female pups shortly after birth to help standardize and enhance pup growth.

2. Care should be exercised in limiting any contact between animals in quarantine and animals released from quarantine, especially those involved in a study.

3. Animals that appear sick or litters with several sick animals should not be released for study.

7.2.3 Documentation (WAT forms 1 through 6)

1. Standard forms are completed and form part of the permanent record for the study. The following standard forms are used:

   a. Animal Order Form (WAT form 1)
   b. Daily Observation Form (WAT form 2)
   c. Animal Study Identification (WAT form 3)
   d. Animal Weight and Identification Record (WAT form 4)
   e. Animal Health Evaluation and Quarantine Release Form (WAT form 5)
   f. Animal Scale Calibration Form (WAT form 6)

7.3 COMPOUND PREPARATION AND ADMINISTRATION (STEP 2)

7.3.1 Operations

1. Unless otherwise specified, solvent or vehicle selection is based upon the Level 1 procedures manual. Solubility testing is recommended prior to initiation of animal testing. Solvents, in order of preference, are: water, corn oil, carboxy methyl cellulose and dimethyl sufoxide.

2. Solids are dissolved or suspended in the appropriate vehicle. Insoluble solids are usually suspended in water 24 hours in advance of dosing to permit water soluble material to leach into the water at room temperature. Alternate solvents are specified in the Level 1 manual.

3. Solids generally are tested without processing. Samples may be ground with a mortar and pestle if the suspension does not pass through a 24-gauge gavage needle.

4. Aqueous liquids are concentrated 4- to 10-fold by lyophilization.

5. Nonaqueous liquids are used without preparation. Resin extracts, while not often tested, should be solvent exchanged to dimethylsulfoxide. Animal solvent controls may be advisable.

6. Animals are weighed within 24 hours of dosing for calculation of dosages. Sets of five male and five female pups of comparable size are selected for each dose set. Animal ear tag number is recorded.

7. Calculations for dosing are checked by a second person prior to dosing.

8. Animal ear tags are noted at time of dosing.

9. Similar compound preparation and administration procedures are used for the quantal and quantitative phases of the in vivo rodent toxicity assay.

7.3.2 Quality Control

1. Upon receipt, each sample is given a unique identification number, is described physically, and is stored as specified (or as appears appropriate).

2. All chemical and physical transformations of the sample are described in a manner that allows the final preparation to be related in quantity and/or concentration to the originally supplied sample.

3. Information on test sample solubilization and preparation of dosing stocks is required.

4. Animals will be dosed under a chemical safety hood exhausted through a HEPA filter unit.

5. Dosing stocks, especially suspensions, are well mixed when aliquots are removed for dosing.

7.3.3 Documentation (WAT forms 7 and 8)

1. The responsible technician fills out the following forms (WAT forms 7 and 8) at the time of the assay. Each form is dated and initiated and constitutes a part of the permanent laboratory record of the assay. The forms are:

   A. Solubility Test Form (WAT form 7)
   B. Compound Preparation Form (WAT form 8)

2. Records from the responsible unit doing test sample manipulations are obtained and maintained with the bioassay data record.

3. All dosing and related handling of the animals is recorded on cage cards and standard forms.

7.4 QUANTAL TOXICITY ASSAY AND ANIMAL OBSERVATION (STEP 3A)

7.4.1 Operations

1. Ten weanling mice are used. After quarantine, 21- to 28-day-old animals (five of each sex) are weighed and dosed by oral gavage.

2. Test substance is administered at the rate of 5 g/kg or 5 ml/kg in one, or up to three fractionated doses, within eight hours.

3. The animals are observed for toxic signs at the time of dosing, at frequent intervals during the first day, and once a day thereafter.

4. The observation period extends for 14 days after dosing unless otherwise specified.

5. Animals that die during the observation period are removed from the cage and necropsied.

7.4.2 Quality Control

1. The types of observations required are described in the Level 1 procedures manual and are reproduced in Table 15.

2. The volume of test material should not exceed 0.3 ml at each administration.

3. Animals are maintained with food and water *ad libitum* during the course of the test.

4. The date and time of death (if known) is recorded for animals that die. Necropsies are performed immediately.

5. Animal ear-tag numbers are noted when observations are made.

6. Technicians should be familiarized with the descriptive terms used in making observations.

7.4.3 Documentation (WAT forms 9 and 10)

1. The Animal Study Record form (WAT form 9) is completed.

2. The responsible technician fills out the Observation Record form (WAT form 10) at each observation time point. Each entry is dated and initialed.

7.5 QUANTITATIVE (LD_{50}) TOXICITY ASSAY AND ANIMAL OBSERVATION (STEP 3B)

7.5.1 Observations

1. The quantitative phase of the *in vivo* rodent toxicity assay is performed in a similar manner to the quantal phase except that multiple-dose levels and a solvent control are used. Operations, as discussed in Sections 2, 3, 4 and 6, are followed.

2. A minimum of 50 weanling mice, equally divided by sex, are used. After quarantine, 21 to 28 day-old animals are divided into five groups of ten animals (five of each sex), weighed and dosed.

TABLE 15. PHYSICAL EXAMINATIONS IN ACUTE TOXICITY TEST IN RODENTS

Organ System	Observation and Examination	Common Signs of Toxicity
CNS and somatomotor	Behavior	Change in attitude to observer, unusual vocalization, restlessness, sedation
	Movements	Twitch, tremor, ataxia, catatonia, paralysis, convulsion, forced movements
	Reactivity to various stimuli	Irritability, passivity, anaesthesia, hyperaesthesia
	Cerebral and spinal reflexex	Sluggishness, absence
	Muscle tone	Rigidity, flaccidity
Autonomic nervous system	Pupil size	Myosis, mydriasis
	Secretion	Salivation, lacrimation
Respiratory	Nostrils	Discharge
	Character and rate of breathing	Bradypnoea, dyspnoea, Cheyne-Strokes respirations, Kussmaul breathing
Cardiovascular	Palpation of cardiac region	Thrill, bradycardia, arrhythmia, stronger or weaker beat
Gastrointestinal	Events	Diarrhea, constipation
	Abdominal shape	Flatulence, contraction
	Feces consistency and color	Unformed, black or clay colored
	Perianal region	Soiled
Skin and fur	Color, turgor, integrity	Reddening, flaccid skinfold, eruptions, piloerection
Mucous membranes	Conjunctiva, mouth	Discharge, congestion, hemorrhage, cyanosis, jaundice
Eye	Eyeball	Exophthalmus, nystagmus
	Transparency	Opacities
Others	Rectal or paw skin temperature	Subnormal, increased
	General condition	Abnormal posture, emaciation

3. Test substance is administered by oral gavage in graded doses usually at the rate of 1.0, 0.5, 0.1, and 0.05 g/kg or ml/kg. Dilutions are prepared using the appropriate solvent. A group of animals is also dosed with the same volume of solvent used in the test material dosing.

7.5.2 Quality Control

1. Quality Control procedures discussed in Sections 7.2, 7.3, 7.4 and 7.6 are followed.

2. Care is taken to identify the dose-level administered to each group of animals.

3. Care is taken to physically separate groups of animals based upon administered dose.

7.5.3 Documentation (WAT forms 9 and 10)

1. Documentation procedures discussed in Sections 7.2, 7.3, 7.4 and 7.6 are followed.

2. The Animal Study Record form (WAT form 9) is completed.

3. The responsible technician fills out the Observation Record form (WAT form 10) at each observation time point. Each entry is dated and initialed.

7.6 GROSS NECROPSY (STEP 4)

7.6.1 Operations

1. Animals are weighed; when found dead during the study period or at the time of sacrifice at the end of the study.

2. Animals that die during the observation period undergo gross necropsy.

3. All animals that survive the observation period also undergo gross necropsy.

4. Organ systems are observed for gross lesions, masses, and discolorations.

5. Questionable findings are brought to the attention of the study director or necropsy personnel.

7.6.2 Quality Control

1. Technicians should be trained to perform rodent necropsies and should be familar with descriptive terminology to be used.

2. Animal ear-tag numbers are noted at time of animal weighing and necropsy.

7.6.3 Documentation (WAT form 11)

1. A Necropsy Report Form (WAT form 11) is completed for each animal necropsied. It should be signed and dated by the technicians.

7.7 DATA EVALUATION (STEP 5) (WAT FORMS 12 AND 13)

Quantal Assay

If no mortality occurs in the quantal study, no further studies are performed with the test substance and the LD_{50} should be reported as greater than 5 ml/kg or 5 g/kg. The test material is ranked as having nondetectable toxicity (ND) at the maximum applicable dose (MAD). Effluent samples which produce harmful effects *in vivo* and do not result in deaths should be noted in the results summary. Such observations are difficult to quantitate but provide insight into the sublethal effects of a sample on rodents. Further investigations may be recommended from observation of nonlethal toxic effects. Initial and final average body weights of the dosed groups are determined and recorded. Gross observations of the necropsies are made and summarized in the report.

Test results from the Quantal Assay are summarized on WAT form 12. Assay information to be entered includes animal number, initial weight, final weight, toxic signs and gross necropsy findings.

Quantitative Assay

If one or more animals die from causes related to the test material, a quantitative assay is performed.

The LD_{50} is calculated by the method of Litchfield and Wilcoxin (1) or other suitable method (e.g. PROBIT) for LD_{50} determination. If the data are not suitable for calculation of a precise LD_{50}, i.e., total mortality occurs at the lowest dose, an estimate of the LD_{50} could be made or the LD_{50} could be expressed as less than 0.05 ml/kg or 0.05 g/kg. Occasionally, it may be necessary to use higher dosages, lower dosages or another series of intermediate dosages depending on the initial results.

The calculated LD_{50} value is used to rank the test sample. Evaluation criteria for toxicity categorization are presented in Table 16.

Observations are made and recorded daily on all animals for the 14-day period. As in the quantal phase, no attempt is made to quantitate or rank the observations. The average animal body weight of each group is determined initially and at the termination of the experiment. The average weights, and the weights as fractions of the control are reported for each dose level. Necropsy observations are recorded and reported.

As in the Quantal Assay, WAT form 12 is used to summarize data in the Quantitative Assay. One form is filled out for each dose level or control. In the Quantitative Assay, survival data is summarized on WAT form 13, LD_{50} Determination Data Form.

TABLE 16. ACUTE IN VIVO RODENT TOXICITY ASSAY EVALUATION CRITERIA

Toxicity	Solids (LD_{50} in g/kg)	Liquids (LD_{50} in ml/kg)
High	<0.05	<0.05
Moderate	0.05 to 0.5	0.05 to 0.5
Low	0.5 to 5	0.05 to 5
Not Detectable	>5	>5

7.8. REFERENCES

1. Litchfield, J.T. and F. Wilcoxon. A Simplified Method of Evaluating Dose-Effect Experiments. J. Pharmac. Exp. Ter. 96:99-113, 1949.

7.9 STANDARD FORMS FOR THE IN VIVO RODENT TOXICITY ASSAY

The following set of data recording and standard procedure documentation forms are adequate to provide the necessary information to audit the EPA Level 1 *in vivo* rodent toxicity assay (WAT).

WAT FORM 1. ANIMAL ORDER FORM

Purchase Request # ______________________ /Purchase Order #______________________

Client ______________________ Project # ______________________

Authority ______________________

Date ______________________

ANIMAL HOLDING REQUIREMENTS

Supplier ______________________

Species ______________________

Strain ______________________

Number of Animals ______________________

Total Males ______________________ Total Females ______________________

Age/Weight Specifications ______________________

Estimated Date Required ______________________

Estimated Date of Delivery ______________________

Quarantine Requirements [Min.] ______________________

Room Requirements ______________________

Type Study ______________________

Type Housing ______________________

Number Animals/Cage ______________________

Holding Period: From ______________________ to ______________________

Other: ______________________

Form Completed By ______________________

LBI-0330 R 10/80

WAT FORM 2. DAILY OBSERVATION FORM

Daily Mortality/Observation **Room No./Ward:**

Mo.-Dy.-Yr.	Temp.	Time	Projects	Observations	Initials

LBI-0503 R 2/81

WAT FORM 3. ANIMAL STUDY IDENTIFICATION FORM

Sponsor ____________________ **Study Design** ____________________

Project # ____________________ **Animal PO #** ____________________

Compound ID ____________________ **Strain/Species** ____________________

Genetics Assay # ____________________ **Reference** ____________________

LBI-0443 2/79							

WAT FORM 4. ANIMAL WEIGHT RECORD

Sponsor ______ **Animal PO. No.** ______ **Scale No.** ______

Project No. ______ **Strain/Species** ______ **Date** ______

Animal No.	Sex	Gm. Wt.	Room No.	Animal No.	Sex	Gm. Wt.	Room No.

$\bar{x}$ Weight ______ **Upper Limit [x 1.15]** ______ **Lower Limit [x .85]** ______

Initials ______

LBI-0601 10/79

WAT FORM 5. ANIMAL HEALTH EVALUATION

ANIMAL HEALTH EVALUATION

____

____

Breeder ____________________ **PO No.** ____________________

Sex-Strain-Species ____

____________________ **Quarantine** ____________________

Project No. ____________________ **Study Design** ____________________

Animal Observations

1 **Weight** ______________________________
2 **Respiration** ______________________________
3 **Haircoat** ______________________________
4 **Feces** ______________________________
5 **Eyes** ______________________________
6 **Behavior** ______________________________
7 **Other** ______________________________

Animals Released for Study[Signature and Date] ______________________________

Animals Not Released for Study*[Signature and Date] ______________________________

***If not released for study, explain why and action taken:** ____________________

____

____

____

____

____

LBI-0504 5/79

WAT FORM 6. SCALE TARE AND CALIBRATION SHEET

Scale ID	Ref. Weights	Trials	Action Taken	Date	Initials

LBI-0470 2/79

WAT FORM 7. SOLUBILITY TEST

Client/Project# ______________________ / ______________________

Test Article/Batch# ______________________ / ______________________

Assay# ______________________

Physical Characteristics of Test Article ______________________

____

Solvent/Lot#	Solvent Volume	Test Article Weight	Final Solution Volume	Final Concentration	Description of Solubility

Date/Time/Initials ____

LBI-0857 10/81

WAT FORM 8. COMPOUND PREPARATION SHEET

ANIMAL GENETICS - COMPOUND PREPARATION SHEET

Sponsor ______________________ **Study Design [protocol]** ______________

Project# ______________________ **Compound Log Book Ref.** ______________

Test Article [batch#] ______________________

Vehicle + Lot# ______________________

Safety# ____________ **Assay#** ____________ **Scale#** ____________

Physical Characteristics of Test Article [T.A.] ______________________

____

Dose Level	Container Weight	Container + T.A.	Total Wt. T.A.	Total Vehicle	Stock Conc.

Kind of Container Used

____

____

Dose Level	Initial Stock Conc.	Vol. from Initial Stock	Volume Diluent	Final Stock Conc.	Amount to Deliver Males	Amount to Deliver Females	Physical Description of Solution

Comments ______________________

____

____

Preparer: Date - Time - Initials ______________________

____

LBI-0858 10/81

WAT FORM 9. ANIMAL STUDY RECORD

ANIMAL STUDY RECORD **STUDY DESIGN** ________________

Sponsor ________________ **Project No.** ________________

Test Article ________________ **Genetics Assay No.** ________________

Vehicle ________________ **Reference** ________________

Dose Initiation ________________ **Breeder** ________________

Dose Termination ________________ **Purchase Order No.** ________________

Test Article Room No. ________________ **Control Room No.** ________________

Article							
Vehicle							
Dose							
Concentration							
Route of Admin.							
Vol./Animal/ Day							

TEST ARTICLE ADMINISTRATION

Date							
Time							
Initials							
Group							

NEGATIVE CONTROL ADMINISTRATION

Date							
Time							
Initials							
Group							

POSITIVE CONTROL ADMINISTRATION

Date		
Time		
Initials		
Group		

COLCHICINE ADMINISTRATION

Mate Date							
Tech. Initials							
Sep. Date							
Tech. Initials							
Sac. Date							
Tech. Initials							

LBI-0175 R 9/81

WAT FORM 10. ANIMAL STUDY OBSERVATION SHEET

ANIMAL STUDY OBSERVATION SHEET **STUDY DESIGN** ____________________

Sponsor ______________________________ **Project Authorization** ______________________________

Compound ID ______________________________ **Genetics Assay No.** ______________________________

Date	Time	Room No.	Observations

LBI-0176 R2/79

WAT FORM 11. NECROPSY REPORT FORM

Animal Number:	Birth Date:	Contract or Project:
Species:	Arrival Date:	Investigator(s):
Sex:	Death Date:	
Age:	Death Time:	
		Inoculum:
		Inoculation Date:

Pertinent Experimental and Clinical Data:

Animal weight at necropsy: ____________________

GROSS NECROPSY: Date:____________, Time:____________, Initials:____________

Description of gross lesions and additional comments:

OPTIONAL

Tissues submitted to:
Microbiology Virology Ultrastructure Hematology Histopathology Frozen

OPTIONAL

Photography -

Tissues cut by: ____________, date: ____________, # capsules: ____________

WAT FORM 12. WAT ASSAY TOXICITY DATA FORM

Sample Identity: ____________________

Assay No.: ____________________

Test Date: ____________________

Animal Strain: ____________________

Quantal ____________________

Quantitative ____________________

Dose Level ____________________

Vehicle ____________________

Animal No.	Initial Weight gm	Initial Weight gm	Toxic Signs[a]	Necropsy Findings

Male

Males: No. Dead/No. Live _____ Initial Weight = ± gm (SD)
Final Weight = ± gm (SD)

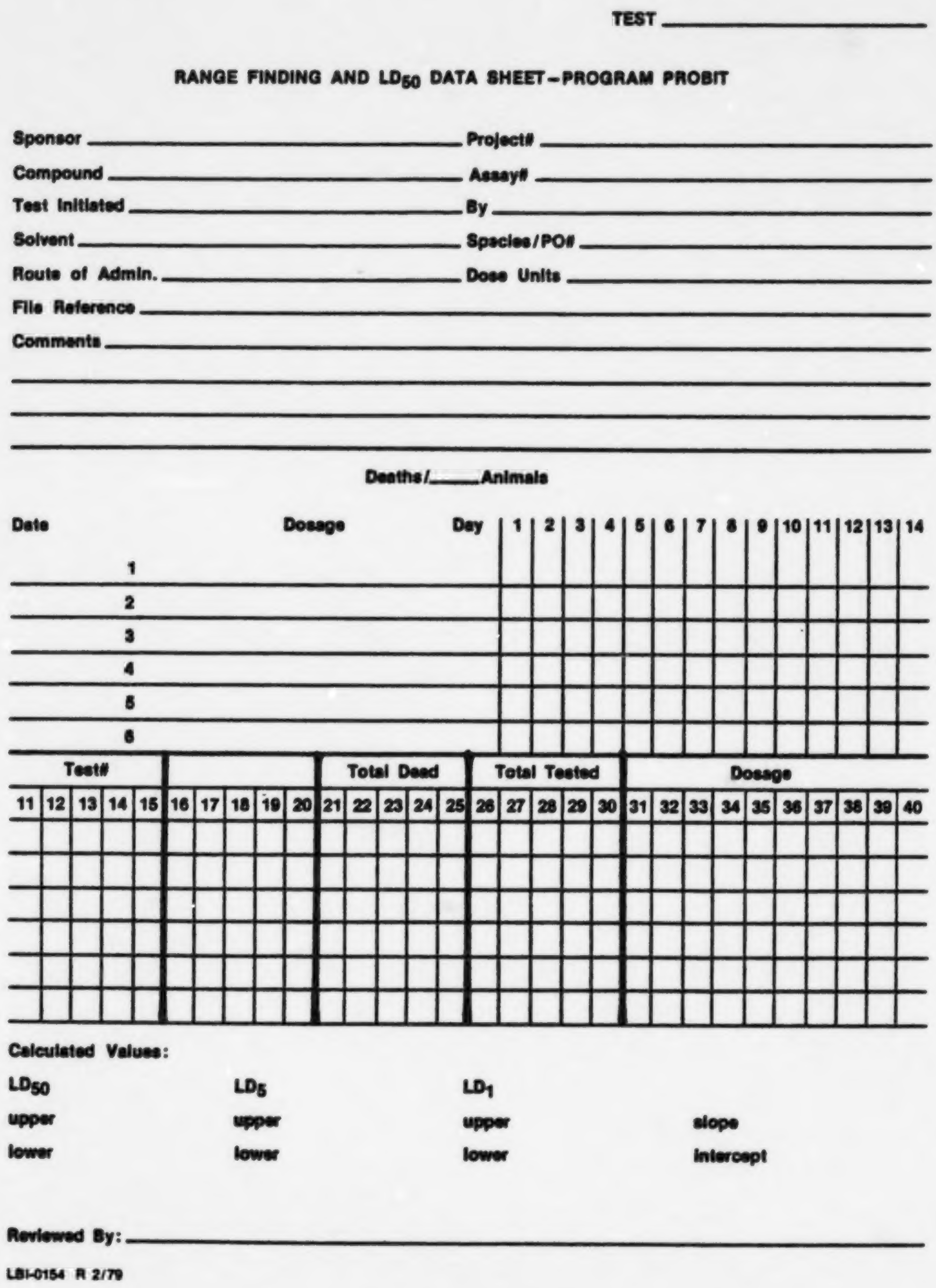
Female

Females: No. Dead/No. Live _____ Initial Weight = ± gm (SD)
Final Weight = ± gm (SD)

---

[a] Toxic signs include death before the observation period.
[b] NTS = No Toxic Signs
[c] NSL = No Significant Lesions

WAT FORM 13. RANGE FINDING AND LD_{50} DATA SHEET-PROGRAM PROBIT



TEST ____________________

RANGE FINDING AND LD_{50} DATA SHEET–PROGRAM PROBIT

Sponsor ____________________ Project# ____________________
Compound ____________________ Assay# ____________________
Test Initiated ____________________ By ____________________
Solvent ____________________ Species/PO# ____________________
Route of Admin. ____________________ Dose Units ____________________
File Reference ____________________
Comments ____________________

Deaths/_____Animals

Date		Dosage	Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	1																
	2																
	3																
	4																
	5																
	6																

Test#										Total Dead					Total Tested					Dosage									
11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40

Calculated Values:

LD_{50}	LD_5	LD_1	
upper	upper	upper	slope
lower	lower	lower	intercept

Reviewed By: ____________________

LBI-0154 R 2/79